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# Periodontitis and Systemic Inflammation:

# Exploring the nature of the association.

Thesis submitted by

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**Abstract**

Periodontitis has been associated with elevated inflammatory markers in otherwise healthy populations. However the nature of this association has not been determined. The aim of this work was to establish whether or not periodontitis causes systemic inflammation.

A first pilot intervention trial indicated that standard periodontal therapy, in a cohort of 94 individuals suffering from severe generalized periodontitis, produced a 0,5 mg/L decrease in serum CRP concentration 6 months after therapy. This decrease was affected by the degree of clinical periodontal response and carriage of specific polymorphisms in inflammatory genes (IL-1A, IL-6).

Periodontal treatment, on the other hand, produced a moderate acute phase response of one week duration [10-fold increase in CRP ($P<0.001$)]. Carriage of rare alleles in the CRP gene was associated with a greater acute CRP response to periodontal therapy after correcting for conventional cardiovascular and inflammatory factors.

Standard (SPT) and an intensive periodontal therapy (IPT, including local delivery of antimicrobials) resulted in a significant reduction in serum CRP compared to an untreated control 2 months after treatment ($0.5\pm0.2$ mg/L, $P=0.030$ and $0.8\pm0.2$ mg/L, $P=0.001$ respectively) in a randomized controlled trial involving 65 healthy subjects with severe generalized periodontitis. The IPT group showed also a decrease in total cholesterol and LDL-cholesterol.

These data were confirmed by the results of a second randomized trial where a cluster of inflammatory and metabolic parameters were evaluated at baseline, 1, 2 and 6 months after either a SPT or IPT regimen. IPT patients showed significant reductions in inflammatory markers at one ($p=0.0406$) and two ($p=0.0060$) months together with an improved metabolic state (2-6 months reduction in lipid markers $p=0.0320$ and $p=0.0432$ respectively).

Periodontitis causes an increased reversible systemic inflammatory burden and an intensive therapy regimen is more effective in re-establishing a more favourable systemic homeostasis.

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## Abbreviations List

**95%CI** – 95% Confidence Interval

**ACTH** – Adrenocorticotrop Hormone

**AGE** - Advanced Glycation End-products

**AHA** – American Heart Association

**ANCOVA** – Analysis of Covariance

**ANOVA** – Analysis of Variance

**ApoE** –Apolipoprotein-E

**APP** – Acute Phase Protein

**APR** – Acute Phase Response

**ARIC** – Atherosclerosis Risk In Communities

**BoP** – Bleeding on Probing

**Ca++** - Calcium++

**CABG** - Coronary Artery Bypass Graft

**CAL** – Clinical Attachment Level

**CDC** – Centre for Diseases Control

**CEJ** – Cemento Enamel Junction

**CHD** – Coronary Heart Disease

**CMV** – Cyto-Megalo-Virus

**Cox** – Ciclo-oxygenase

**CPTIN** – Community Periodontal Treatment Index Need

**CRM** - Certified Reference Material

**CRP** – C-Reactive Protein

**CSD** – Cerebro Ischaemic Disease

**CVS** – Cardiovascular

**CV** – Coefficient of Variation

**CVD** – Cardiovascular Disease

**DNA** – DeoxyriboNucleic Acid

**ECIC** – Eastman Clinical Investigation Centre

**EDI** – Eastman Dental Institute and Hospital

**EDTA** - Ethylene Diamine Tetra Acetate

**ELISA** – Enzyme Like ImmunoSorbent Assay

**FcγRI** - Fcg Receptor I

**FcγRII** - Fcg Receptor II

**FMBS** – Full Mouth Bleeding Score

**FMPS** – Full Mouth Plaque Score

**HDL** – High Density Lipoprotein

**HIV** – Human ImmunoDeficiency Virus

**HPA** - Hypothalamic-Pituitary-Adrenal

**HSP** – Heat Shock Protein

**HSV** – Herpes Simplex Virus

**IgG** – Immunoglobulin G

**IGLS** - Iterative Generalized Least Square

**IL-1** – Interleukin-1

**IL-10** – Interleukin-10

**IL-1A** - Interleukin-1A gene

**IL-1β** - Interleukin-1b

**IL-1B** - Interleukin-1B gene

**IL-1Ra** – Interleukin-1 Receptor Antagonist

**IL-6** – Interleukin-6

**IQR** – Inter Quartile Range

**IR** - Insulin Resistance

**IRS** - Insulin Resistance Syndrome

**LDL** – Low Density Lipoprotein

**LPS** – Lipopolysaccaride

**MI** – Myocardial Infarction

**MLM** – Multi Level Modelling

**NFkB** – Nuclear Factor kB

**NHANES** - National Health and Nutrition Epidemiologic Study

**NO** - Nitric Oxide

**NPPD** – Number of Periodontal Pockets

**NSAID** – Non Steroidal Anti Inflammatory Drug

**OD** – Optical Density

**OR** = Odds Ratio

**PCR** – Polymerase Chain Reaction

**PLBW** - Preterm low birth-weight

**PLGA** - PolyLactic-co-Glycolic Acid

**PMN** – Poly-morpho-nuclear

**PPD** – Probing Pocket Depths

**PRR** – Pattern recognition receptor

**REC** – Recession

**SD** – Standard Deviation

**sICAM-1** – soluble Inter-cellular Adhesion Molecule-1

**TB** - Tubercolosis

**TNF-A** – Tumor Necrosis A gene

**TNF-α** - Tumor Necrosis Factor-a

**t-PA** – tissue-Plasminogen Activator

**UCL** – University College London

**UNC** – Universal North Carolina

**VLDL** –Very Low Density Lipoprotein

**WBC** – White Cell Count

**List of publications**

D'Aiuto,F, Parkar,M, Andreou,G, Brett,PM, Ready,D, and Tonetti,MS (2004). Periodontitis and atherogenesis: causal association or simple coincidence? *J Clin Periodontol* 31(5):402-411.

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# CONTENTS

## LIST OF FIGURES

PAGE

## LIST OF TABLES

PAGE

# CHAPTER 1.

# INTRODUCTION

## 1.1 SYSTEMIC INFLAMMATION (ACUTE PHASE RESPONSE)

### 1.1.1 DEFINITION

Inflammation represents a highly conserved and complex reaction of the organism to a series of pathological insults such as infection, tissue injuries, tissue infarction, surgery or malignancies (Baumann & Gauldie 1994). Inflammation can be clinically defined as the presence of rubor (redness), tumor (swelling), calor (heat), dolor (pain) eventually leading to functio laesa (loss of function). Systemic inflammation encompasses a series of physiologic changes (plasma proteins levels, behavioural, biochemical and nutritional changes) produced by a pathological insult that are usually distant from the site of application of the inflammatory stimulus (Table 1) (Baumann & Gauldie 1994).

**Table 1** Acute Phase Reaction

| ACUTE PHASE PHENOMENA | |
|---|---|
| Neuro-endocrine Changes | Fever, somnolence and anorexia<br>↑ ACTH and cortisol<br>↑ Vasopressin<br>↑ Circulating catecholamines |
| Hemapoietic Changes | Leucocytosis<br>Thrombocytosis<br>Anemia |
| Metabolic Changes | ↓ gluconeogenesis<br>↑ hepatic lipogenesis<br>↑ lipolysis in adipose tissue<br>Osteoporosis<br>↑ lipoprotein lipase activity (muscle and adipose tissue)<br>Cachexia |
| Hepatic Changes | ↑ production of plasma proteins (CRP, Fibrinogen, Serum Amyloid A, Ceruloplasmin, Haptoglobin....)<br>↑ inducible NO synthase, heme oxygenase, manganese superoxide dismutase, tissue inhibitor of metalloproteinase-1 |
| Biochemical Changes | ↓ Zinc<br>↓ Fe<br>↓ Cu<br>↑ plasma retinol and glutathione |

These mechanisms have been classically referred to as the acute phase response (APR) even though they are present in both acute and chronic inflammatory conditions

(Baumann & Gauldie 1994; Gabay & Kushner 1999).The organism undertakes such changes to remove or contain the injury; this is accomplished by isolating and destroying the infective organisms, removing any harmful molecules, and/or activating the repair process. APR is a non specific rapid response to a variety of stimuli and it is part of the innate immunity. Complex organisms produce it in response to injury via cellular and humoral mechanisms. Over the ages, the evolution of this response has allowed complex organisms to protect themselves from infections and a series of external noxious agents thanks to the rapidity by which the APR manifests. The induction of such defensive mechanisms seems to depend upon recognition of very broad molecular patterns in pathogens by highly preserved pattern recognition receptors (PRR) present on defensive cells like macrophages and polymorphonuclear leucocytes. All multicellular organisms are believed to have some form of innate host defences. Plants resistance to diseases and defence against pathogens includes the action of highly conserved protein modules found also in vertebrates. In mammals similar transcription factors and recognition protein are also involved in the control of innate immune responses (NFkB family, Toll domain) (Medzhitov & Janeway, Jr. 1996; Medzhitov & Janeway, Jr. 1998; Medzhitov & Janeway, Jr. 2000).

Highly preserved mechanisms as those based on pattern recognition molecules (i.e. against bacterial LPS) are examples of the protective effects of the APR within the human response to endotoxemia (Baumann & Gauldie 1994; Medzhitov et al. 1998). APR further improves survival after an injury such as a trauma, burn or an ischemic necrosis (myocardial infarction) (Ebersole & Cappelli 2000; Pepys & Hirschfield 2001; Liuzzo et al. 1999). APR is not the only defensive reaction that the organism can mount as part of the homeostatic response to injury: innate cellular responses also play a significant role.

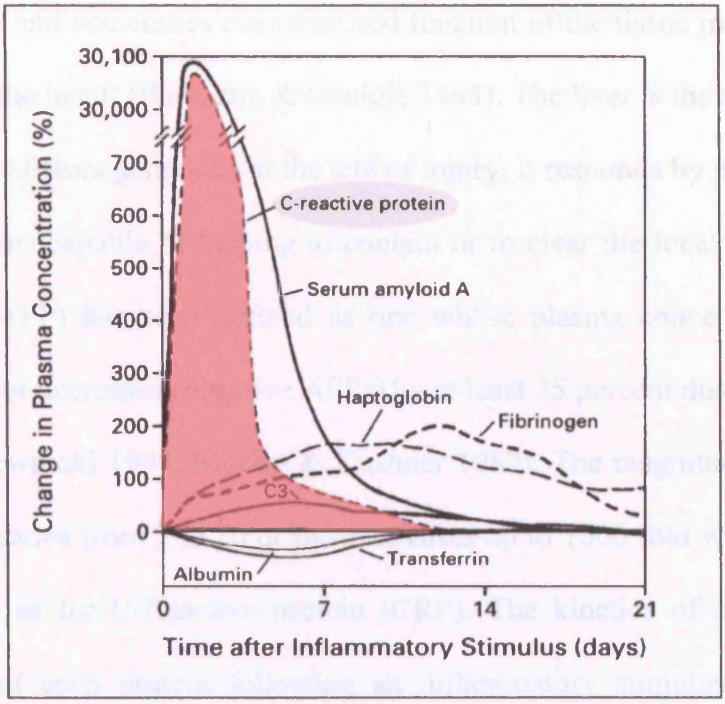


**Figure 1** Acute-Phase Proteins Kinetics
Adapted from (Gabay & Kushner 1999)

### 1.1.2 Acute-Phase Mediators

The APR has been primarily associated with the systemic response of the organism to an insult. This reaction, however, is initiated at the site of injury where activated cellular components produce a co-ordinated cascade of signalling peptides (cytokines). Some of these eventually gain access to the circulation and are thus able to produce distant effects (amplification and modulation of the APR).

At the cellular level, macrophages and monocytes are the major sources of inflammatory cytokines involved in the APR (Koj 1996). Cytokines have multiple targets; their regulatory activities are exerted via specific receptors that activate intracellular signalling pathways which promote nuclear gene transcription, changes in the expression signature and eventually change in the cellular phenotype (Lin et al. 2000).

At the vascular level the action of many cytokines leads to increased permeability and vascular leakage to facilitate the host response towards the injury. This results in

oedema, redness and sometimes compromised function of the tissue involved according to the nature of the insult (Baumann & Gauldie 1994). The liver is the main target of the inflammatory mediators generated at the site of injury; it responds by producing a series of plasma proteins capable of helping to contain or to clear the local insult. An acute-phase protein (APP) has been defined as one whose plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25 percent during inflammation (Kushner & Rzewnicki 1994; Morley & Kushner 1982). The magnitude in rise of each plasma protein varies from 2 to 10 or in some cases up to 1000 fold within 24-48 hours from the injury as for C-Reactive protein (CRP). The kinetics of changes in serum concentrations of each protein following an inflammatory stimulus are presumably associated with its function and have been widely studied and used reliably for monitoring the induction or resolution of the APR itself (Figure 1). Although plasma proteins seem to increase according to an exponential curve, not all individuals show uniformity in such increase. These differences might be due to different pattern of production of specific cytokines (Kushner 1991; Kushner 1993; Kushner et al. 1995).

The synthesis of the APPs is regulated not only by molecular mediators (cytokines) but also by hormones (i.e glucocorticoids) and growth factors. Arbitrarily we can consider i) Interleukin-1 (IL-1) type cytokines [IL-1, Tumor Necrosis Factor-α (TNF-α)] considered local rapid mediators and inducers of the systemic response, ii) Interleukin-6 type cytokines (IL-6, interleukin-11 etc) responsible for the systemic acute-phase changes described above and iii) glucocorticoids and growth factors (including insulin) mainly involved in modulating the APR. Cytokines in particular stimulate the plasma proteins gene-expression, while glucocorticoids and growth factors modulate cytokine activity. When these two opposing mechanisms are balanced then the organism successfully resolves the APR by clearing the insult and/or by repairing any damage.

The effect of each cytokine depends ultimately upon the context in which it is working (Sporn 1997). IL-6 is widely known as the primary inducer of the APR, acting directly at the hepatocyte level by stimulating or inhibiting the synthesis rate of each protein at the transcriptional level (Baumann & Gauldie 1994; Ganapathi et al. 1988a; Ganapathi et al. 1988b; Kishimoto et al. 1992; Mackiewicz et al. 1991). Its activity, however, seems to be dependent upon the nature of the stimulus. Experimental models to study the initiation of the APR in which animals where rendered incapable of producing IL-6 (knock-out), indicated that only after tissue injury (turpentine injection, sterile inflammatory stimulus) and not infection (LPS injection) a normal systemic APR developed (Fattori et al. 1994). This observation indicates that during an infectious process besides IL-6 other peptides (IL-1 and TNF-α) act independently as potent systemic acute phase inducers; some cytokines initiate and amplify the response, others sustain or attenuate it, and some of them cause it to resolve. The inter-relationship between IL-6 and other inducing cytokines (IL-1, TNF-α) together with the hypothalamic-adrenocortical axis stimulation completes the complex web of interactions involved in the APR. It is rare that *in vivo* cells are stimulated only by one cytokine while presumably we should expect more an interchange of additive or inhibitory activities exerted by all mediators involved.

### 1.1.3 INTERLEUKIN-6

IL-6 is a pleiotropic peptide of 26 kDa that shows a variety of actions on the immune system, haemostasis control and neuroendocrine system (Barton 1997). It was originally known as hepatocyte-stimulating factor (HSF) but now IL-6 is considered to be primarily responsible for the regulation of production of acute phase proteins (Barton 1997; Ritchie & Fuller 1983). Unlike the other cytokines that act primarily as auto/paracrine factors and their circulating levels are un-related with their patho-physiological role, IL-6 acts at distant sites as hormones do.

During inflammation it is generally believed that different cytokines are produced following a specific sequence (TNF-$\alpha$ $\rightarrow$ IL-1$\beta$ $\rightarrow$ IL-6) (van Deventer et al. 1990). Interleukin-6 shows an inhibitory activity on the secretion of both TNF-$\alpha$ and IL-1$\beta$ (Schindler et al. 1990); IL-6 also activates the hepatic synthesis of APPs (Heinrich et al. 1990), and stimulates the hypothalamic-pituitary-adrenal axis (HPA) (Lyson & McCann 1991) to help control the inflammation. This is why IL-6 can be considered both as a pro-inflammatory and an anti-inflammatory molecule. The latter activity seems to be essential in survival and response to acute infection (Xing et al. 1998).

IL-6 is one of the principal mediators also of the clinical manifestations of tissue injury, including fever, cachexia, and elevation in resting metabolic rate (Tsigos et al. 1997b; Tsigos et al. 1997a). When injected in healthy volunteers, leukocytosis, thrombocytosis, decreased plasma levels of albumin, reductions in serum total cholesterol, apolipoprotein B and triglyceride levels were observed within 24 hours (Papanicolaou et al. 1998). IL-6 type cytokines play also a profound role in skeletal homeostasis since they promote osteoblasts/osteoclasts development (Manolagas 1995).

Besides immune accessory cells (such as monocytes, macrophages etc) which are the main reservoir, IL-6 is produced by many non-immune cells (such as osteoblasts, keratinocytes, etc) (Papanicolaou et al. 1998). Adipocytes of healthy individuals can

produce great quantities of this peptide such as to account for up to 30% of total circulating IL-6 (Mohamed-Ali et al. 1997; Mohamed-Ali et al. 1998). IL-6 promotes megakaryocyte maturation and B cell differentiation even though it is not essential for B cell development (Baatout 1996; Fattori et al. 1994). Plasma concentrations of this cytokine increase rapidly after sepsis and the magnitude of its increase correlates well with the extent, duration and severity of the stimulus (Barton 1997).

IL-6 actions are mediated through a cellular receptor (130 kDa) which once activated induces a cascade signalling pathway (kinases, transcription factors) (Hallek et al. 1997) and a soluble circulating receptor (Papanicolaou et al. 1998). Notably IL-6 synthesis has been thought for some years to be affected by a diurnal variation (Chrousos 1995) however, new evidence is suggesting that the increased concentrations of IL-6 reported in the afternoon perhaps are a consequence of the sampling (IV catheter) procedure referring to a possible vascular damage and local site of inflammation (Haack et al. 2002) rather than a different synthesis rate.

During acute stimulation IL-6 activates the HPA axis by acting primarily on the corticotropin-releasing hormone neuron. (Nishimura et al. 2000) and resulting in elevated plasma levels of adrenocorticotropin hormone (ACTH) and consequently cortisol (Tsigos et al. 1997a). Glucocorticoids' synthesis inhibits production of interleukin-6 in vitro and in vivo (Breuninger et al. 1993; Mastorakos et al. 1993) acting as a negative feedback control.

Located on the short arm of chromosome 7, the IL-6 gene consists of 5 exons and 4 introns and has a fairly complex transcriptional regulation (Sehgal 1992). The 5' and 3' flanking regions have been reported to be polymorphic (Fishman et al. 1998). A biological activity of a specific base exchange (G/C -174) has been recently reported in a population of individuals suffering from systemic-onset juvenile rheumatoid arthritis. G allele carriers (common allele) showed higher spontaneous and induced (IL-1, LPS)

IL-6 concentrations when compared with individuals homozygous for the rare allele (Fishman et al. 1998). Mechanistic explanation of such differences is still lacking and controversial since other reports have shown an opposite genotype effect (Matsusaka et al. 1993).

### 1.1.4 C-Reactive Protein

CRP was first discovered in 1930 and named after the capacity to bind the C-polysaccharide of *Streptococcus pneumoniae* during the acute phase of infection. Since then and thanks to its sensitivity to inflammatory stimuli, its fast response and wide range of observed concentrations it has been considered the prototype acute phase marker (Hirschfield & Pepys 2003; Pepys & Baltz 1983; Pepys 1981). CRP is a pentameric protein composed of five identical subunits (figure 2). It belongs to a family of highly evolutionary conserved pattern recognition molecules, the pentraxins which are all Ca++ dependent ligand-binding proteins. CRP in particular binds with high avidity to phosphocholine and to a series of other autologous and extrinsic ligands (i.e. lipoproteins, damaged cell membranes, apoptotic cells and FcγRI and FcγRII) (Hirschfield & Pepys 2003; Szalai et al. 1997; Szalai 2002; Szalai & McCrory 2002). When bound to bacteria it is immediately recognized by C1q and subsequently it activates the classical complement pathway miming the opsonization activity of antibodies (Hirschfield & Pepys 2003). Many believe that one of its biological functions is to bridge together innate and adaptive immunity (Szalai et al. 1997). Besides complement activation, CRP actions are predominantly defensive

**Figure 2** CRP pentameric structure
Protein Data Bank (Human C-Reactive Protein 1GNH)(Hirschfield & Pepys 2003)

including enhancement of bacterial opsonization, scavenging of apoptotic cells and modulation of PMN function (Pepys 1981).

In healthy individuals its serum concentrations are below 1mg/l with a $90^{th}$ percentile of 3mg/l (Hirschfield & Pepys 2003). Within 4-5 hours of an intense inflammatory stimulus (surgery or tissue infarction) its concentration increases rapidly reaching its peak within 24-40 hrs (up to 500 mg/l). It is mainly produced by hepatocytes under stimulation of one potent inducer (IL-6), but other local sites of production (such as the stimulated endothelium) have been recently discovered (Gabay & Kushner 1999).

Due to its relatively short but constant plasma half-life (19 hours) CRP serum values are only dependent of the synthesis rate. This important feature enables physicians to use CRP concentrations to monitor the entire course of the systemic inflammatory response from initiation to resolution. CRP does not present diurnal variation and the individual concentration is relatively stable with peaks only during periods of infection or inflammation (Macy et al. 1997). There is no seasonal variation and over 5 years time its concentration self-correlation coefficient is similar to that of cholesterol (0.5). CRP concentrations are therefore a sensible and reliable marker of individual inflammatory burden in presence of organic diseases, infections or other inflammatory conditions. IL-6 concentrations are highly correlated with this marker both in health and disease (de Maat et al. 1996; de Maat & Kluft 2001).

Women tend to have higher concentrations of this marker in physiological conditions (de Maat & Kluft 2001), even though the association between hormone replacement therapy and higher CRP concentration is well established (de Maat & Kluft 2001). Smokers as well as obese individuals show higher CRP serum levels presumably because of local tissue-damage or recurrent infections and increased IL-6 concentrations respectively (de Maat & Kluft 2001; Szalai & McCrory 2002). Finally some medications (e.g. non-steroidal anti-inflammatory drugs, statins) have clearly shown

either a positive or negative effect on the serum level of CRP (de Maat & Kluft 2001; Szalai & McCrory 2002).

Twin and family studies indicate that CRP concentrations are a heritable trait (MacGregor et al. 2004; Pankow et al. 2001). Recent data have indicated that the CRP gene is polymorphic. A polymorphism in the exon 2 of the CRP gene has been described but without any biological activity. A novel polymorphism (+1444C>T) in the 3' untranslated region of the gene encoding CRP has been reported to have functional activity (Brull et al. 2003). Healthy subjects and coronary artery bypass graft (CABG) patients who were homozygous for the T allele were associated with higher basal and stimulated CRP concentrations in male subjects (Brull et al. 2003). However, it is likely that other important genetic influences, such as the –174G>C polymorphism of the IL-6 gene, (Vickers et al. 2002) may also determine the basal and stimulated concentrations of CRP as a consequence of different cytokine concentrations. Polymorphisms in other pro-inflammatory genes (IL-1 mainly) have also been associated with higher CRP concentrations in healthy and diseased population studies. Relatively little, however, is known about the exact mechanisms through which the inflammatory burden and/or systemic response is increased (Berger et al. 2002).

### 1.1.5 APR AND HOMEOSTASIS

APR includes a series of neuroendocrine, haematopoietic, metabolic and hepatic changes, which scientists have interpreted to be presumably defensive. Fever appears during the early stage of the APR and it is thought to be induced by specific cytokines (IL-1 and IL-6) and is accompanied by other neuroendocrine (activation of the HPA-axis) (Chrousos 1995) and behavioural changes (anorexia, somnolence).

Neutrophilia, reduced serum iron with consequent anaemia, increased gluconeogenesis and coagulation activation are also part of the same reaction (Moshage 1997). The acute-phase changes include an increase in the number of circulating leucocytes (polymorphonuclear) and platelets, released both from the circulatory pool to the local sites and resulting from increased production in the bone marrow (Suffredini et al. 1999).

Many of these events are potentially harmful to the organism. An uncontrolled inflammatory reaction to a variety of stimuli can produce more damage than benefits. In the past decade we have witnessed an increased awareness that a long-term exposure to stressful (inflammatory) stimuli may produce disease rather than repair. Inflammatory diseases such as Rheumatoid Arthritis or Periodontal Chronic Infections are prototypic examples. We assume that APR is a defensive mechanism of the organism since the action of each individual mediator we have studied has been thought to be protective. This process however is not a linear cascade of activation of peptides and organs but a series of fine tuned modulating mechanisms which are evoked in order to contain this reaction and protect the host.

Over the years the term "homeostasis" has been referred to a status of general health and equilibrium that the organism seeks during his lifespan. APR represents perhaps one of the most common, rapid and efficient mechanisms through which every organism may accomplish the homeostatic status. According to the entity of the

inflammatory stimulus or sometimes to an individual variability, an acute phase reaction can be different in magnitude even though the pathogenetic mechanisms remain similar. Deviation from normal homeostasis may sometimes produce uncontrolled and potentially harmful conditions such as sepsis or systemic inflammatory response syndrome. The latter encompasses a series of haemodynamic, vascular permeability changes, coagulation defects and multiple organ failure that, when it accompanies severe injuries (trauma or infections), may negatively affect survival (Angele & Faist 2002; Das 2000; Riewald & Ruf 2003).

Over the past decades therefore a series of experimental models to study acute inflammation in humans have been developed. To study the regulation of the synthesis of pro-inflammatory cytokines, for instance, investigators have predominantly adopted in vitro models (stimulation of blood mononuclear cells with LPS, other stimuli or whole blood assay). However it is still not known to what extent the information obtained can be referred to an *in vivo* situation where a more complex web of signalling takes place. To prevent this problem experimental human models of acute inflammation have been developed. The prototype model is the parenteral injection of bacterial endotoxin (LPS) in healthy individuals which causes a sharp increase in all acute inflammatory markers and it is accompanied by classical flu-like symptoms (Suffredini et al. 1995; Suffredini et al. 1999). Drug-induced models have also been developed (vaccination against *S. tiphy* or Yellow fever) (Hingorani et al. 2000; van, I et al. 2002). The only non drug-induced inflammation model reported to date and accompanied by a similar systemic involvement but smaller in magnitude is the strenuous exercise model (Fiuza & Suffredini 2001; Martich et al. 1993; Moldoveanu et al. 2001; Parker & Watkins 2001; Shek & Shephard 1998; Shephard & Shek 1998; Shephard 2002). This line of research aims at the identification if ever possible of individual hypo/hyper-

responders to mild or strong inflammatory stimuli and at the discovery of specific preventive or therapeutic measures for both healthy subjects and patients.

Increased interest in studying the onset and resolution of low-grade inflammatory processes has recently increased especially after the discovery of a possible important pathogenetic role of persistent low-grade inflammation on systemic diseases such as atherosclerosis, diabetes, hypertension (Bautista 2003; Libby et al. 2002; Pradhan et al. 2002).

## 1.2 APR AND SYSTEMIC DISEASES

### 1.2.1 DIABETES

Chronic sub-clinical inflammation, assessed by moderately elevated CRP and IL-6 levels, plays a significant role in the pathogenesis of type-2 diabetes (Festa et al. 2003; Pradhan et al. 2002). Insulin resistance (IR), which represents perhaps the main characteristic of patients in their pre-diabetic state, has been significantly associated with levels of acute phase proteins in response to pro-inflammatory cytokines (Festa et al. 2003). The term IR refers to a diminished ability of insulin to metabolize glucose, producing glucose intolerance and hyperglycaemia. The relationship between insulin resistance and inflammation is bidirectional. Independent of the inflammatory triggering factors a deficit in insulin activity leads to a worsening of a chronic inflammatory state (Fernandez-Real et al. 2003; Fernandez-Real & Ricart 2003). Among all possible risk factors behind the individual inflammatory burden, obesity may play a crucial role. Adipose tissue represents a major source of inflammatory cytokines (IL-6, IL-1Ra, TNF-α) (Fernandez-Real & Ricart 2003). In normal conditions adipose tissue is targeted by a variety of still unknown stimuli. IL-1β for example appears to be a greater inducer than TNF-α or LPS on the production of IL-6 from adipocytes (Mohamed-Ali et al. 1998). This observation suggests a possible role of the adipose tissue in innate immunity. Measures of obesity (e.g. body mass index) are positively correlated with IL-6. IR is negatively correlated with plasma and adipose content of IL-6 (Fernandez-Real et al. 2003). A second possible source of inflammation might be the muscles corporeal mass. Insulin can stimulate IL-6 production after strenuous exercise (Fernandez-Real et al. 2003). Insulin is also one of the major modulators of the cytokine-associated APR (Campos & Baumann 1992; O'Riordain et al. 1995). Its main activity would be to attenuate the IL-6-type cytokines stimulation of liver APR (Campos & Baumann 1992;

O'Riordain et al. 1995). Type-2 diabetic patients therefore, because of a reduced insulin action or concentration, when challenged by various insults mount prolonged acute phase reactions due to the absence of such modulating activity (Fernandez-Real & Ricart 2003).

Crude markers of systemic infection/inflammation such as white cell counts (WBC) have been associated with several components of the insulin resistance syndrome (IRS) (Targher et al. 1996). Plasma insulin was associated with the number of lymphocytes and monocytes in healthy individuals or those suffering from an infection (Targher et al. 1996). Acute infections indeed cause insulin resistance leaving some glucose impairment even after clinical symptoms resolve (Yki-Jarvinen et al. 1989).

Given its haematopoietic activity, IL-6 has been suggested to be the mechanistic link between IR and increased WBC (Fernandez-Real & Ricart 2003). Serum CRP concentration in particular has been significantly associated with measures of IR (Fernandez-Real & Ricart 2003). This negative correlation seems to be independent of obesity (Campos & Baumann 1992).

### 1.2.2 HYPERTENSION

Mechanisms underlying various forms of hypertension are multi-factorial even though Nitric Oxide (NO) seems to play a major role (Manning et al. 2001). This molecule is essential for vascular relaxation due to its antagonistic activity against a potent vasoconstrictor (Angiotensin-II) (Bataineh & Raij 1998). NO production is reduced during hypertension (Leclercq et al. 2002). Systemic chronic inflammation has been associated with high blood pressure in several epidemiological studies (Bautista 2003). Serum CRP concentrations and hypertension seem to be positively associated (Bautista 2003). This association is however highly modulated by the effect of other recognized risk factors such as age, gender and body mass index. IL-6 and TNF-α have also been separately linked to hypertension but the evidence reported in the literature still lacks

control for classical cardiovascular risk factors such as smoking and diabetes (Volpato et al. 2001). The plausible biological mechanism behind this link could be that systemic inflammation produces an imbalance of the normal endothelial contracting and vasodilator factors and activity (endothelial dysfunction). There is for instance considerable evidence of the influential effect of chronic and acute inflammation, defined by serum levels of CRP, and endothelial dysfunction (Bhagat & Vallance 1997; Hingorani et al. 2000; Vallance et al. 1997). An impaired endothelial-dependent dilatation activity has been observed following the action of a series of inflammatory mediators (IL-1, TNF-α, IL-6). In physiologic conditions NO is released by endothelial cells whenever vasoconstrictor, mechanical and/or thrombogenic stimuli are present. NO induces vasodilatation, inhibits platelet aggregation and WBC adhesion to the endothelium (Bautista 2003). A plausible effect of systemic low-grade inflammation, even produced by bacterial endotoxin (Hingorani et al. 2000), is that of directly affecting the endothelial cells and thus of reducing the local availability of vasodilating factors by: i) decrease in intrinsic NO production (Yoshizumi 2004), ii) increase in NO degradation through an elevated production of $O_2$- radicals (Gryglewski et al. 1986), iii) increased production of Cox-derived prostanoids which manifest potent contracting activity (Taddei et al. 1996). More research however is needed to establish whether cytokine elevation in chronic inflammation is the cause rather than the consequence of essential hypertension.

### 1.2.3 ATHEROSCLEROSIS

Inflammation defined by increased concentrations of serum levels of pro-inflammatory cytokines and acute phase reactants has been closely linked with atherosclerosis. Among all the possible associations between APR and systemic diseases perhaps this one represents the most interesting one because of the potential preventive and therapeutic implications.

The word "atherogenesis" has evolved over the years and assumed several meanings. It is not anymore referred just to a mere accumulation of lipids in the intima portion of arterial walls even though increased LDL-cholesterol still represents the major risk factor. It is now widely accepted to consider atherosclerosis an inflammatory disease (Ross 1999). Each stage of the atherosclerotic process from the early fatty streak formation (a leucocytes infiltrate of the vascular wall) to the advanced lesion development and plaque rupture are being considered an inflammatory process at the molecular level (Berliner et al. 1995; Libby et al. 2002)(Figure 3).

In normal conditions the vascular endothelium does not bind any lipoprotein molecules and only upon starting an atherogenic diet animal models have shown that leucocytes adhere and start infiltrating the intimal space. Why the endothelium integrity is impaired remains a matter of debate. The discussion about atherosclerosis' aetiology has been a major research topic for many decades. Various pathogenetic theories have been postulated. The response to injury and shear stress thesis are among them (Libby et al. 2002). The first referred to a potential endothelial harmful activity predominantly of circulating lipoproteins which turned into an enhanced expression of vascular adhesion molecules. This particular stage of the atherosclerotic process is known to always precede leucocytic infiltration within the arterial wall (Libby et al. 2002). The shear stress theory focused instead on the potential damage that changes in the

haemodinamics of the blood flow could produce at specific sites of the vasculature (e.g. bifurcation, aortic sinus).

Advances made in understanding the molecular pathophysiology of the vascular endothelium have underlined the importance of the integrity of the normal vasodilator-vasoconstrictor activities in avoiding the onset of atherosclerosis (endothelial function). Pro-inflammatory cytokines promote the expression of vascular adhesion molecules and the migration of monocytes in the sub-endothelial space. Inflammatory processes

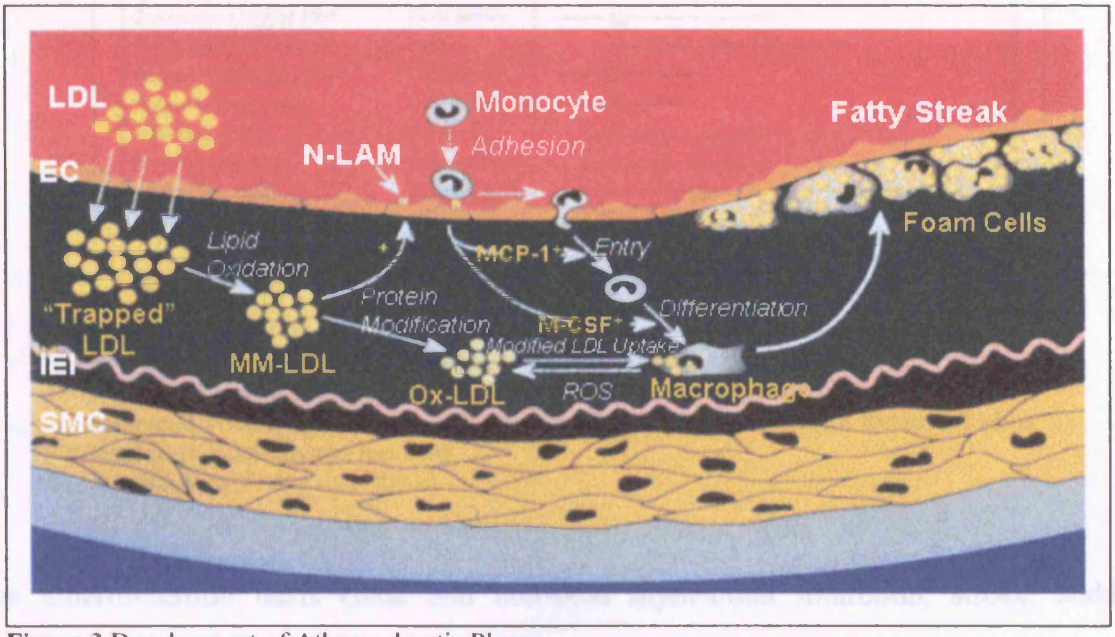


**Figure 3** Development of Atherosclerotic Plaque
Low-density cholesterol (LDL) molecules can infiltrate the endothelium. Once trapped into the intimal space, oxidative processes can modify LDL and produce oxidized-LDL. Monocytes can adhere to the endothelial cell (EC) surface via a series of vascular adhesion molecules and once into the intima space may differentiate in intravascular macrophage. SMC(smooth muscle cells).Macrophages can uptake Ox-LDL and become foam cells.
Source (Berliner et al. 1995)

accompany the evolution of the fatty streak lesion and IL-1, TNF-α and IL-6 are all implicated in the maturation of the macrophages in the local plaque formation up to the foam cell stage. Inflammation furthermore seems to contribute decisively to precipitating the acute thromboembolic complications of the atheroma (Libby et al. 2002).

A large amount of clinical evidence from a series of prospective population studies has clearly confirmed this hypothesis. Elevated levels of inflammatory mediators among apparently healthy individuals predict the occurrence of future cardiovascular events on

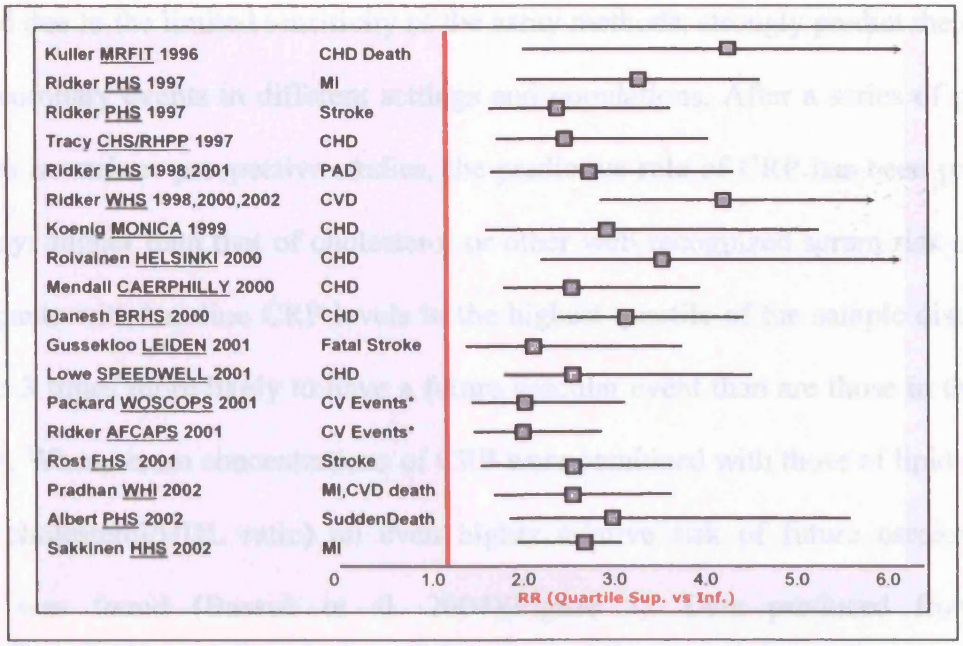


**Figure 4** CRP as predictor for future CVD events in different population studies.
In a series of epidemiological studies one single measure of serum CRP revealed its value as predictor of future CVD. Whenever individuals of the upper quartile were compared with those of the lowest quartile of CRP concentrations a Relative Risk at least double was always reported.
Adapted from (Ridker et al. 2000a)

an atherosclerotic basis (fatal and non-fatal myocardial infarction, stroke, sudden cardiac death). In particular some epidemiological studies have found an increased cardiovascular risk to be associated with increased basal levels of IL-6 and TNF-α (Harris et al. 1999; Ridker et al. 1997). Many classic cardiovascular risk factors (obesity, diabetes and hypertension) correlated well with a cluster of inflammatory markers (CRP, TNF-α, IL-6).

Similarly CRP has been measured across different populations and it has demonstrated to be a better predictor of future cardiovascular events than the classical Framingham risk factors. A meta-analysis showed how this acute phase marker constantly manifested a strong and significant association with future cardiovascular events after correcting for

the confounding effect of other risk factors such as smoking, age, cholesterol, diabetes, hypertension etc (Danesh et al. 2000)(Figure 4).

Mildly increased basal levels of CRP (2-3mg/l), which in the past would have not been detected due to the limited sensitivity of the assay methods, strongly predict the onset of future coronary events in different settings and populations. After a series of post-hoc analyses in various prospective studies, the predictive role of CRP has been proven to be always higher than that of cholesterol or other well recognized serum risk markers. Participants with baseline CRP levels in the highest quartile of the sample distribution are 2 to 3 times more likely to have a future vascular event than are those in the lower quartile. When serum concentrations of CRP were combined with those of lipid markers (Total cholesterol/HDL ratio) an even higher relative risk of future cardiovascular events was found (Bassuk et al. 2004)(Figure 5). Data produced from large interventional randomized trials (statins) indicated that a reduction of cardiovascular mortality and number of events was highly influenced by the presence of a concomitant

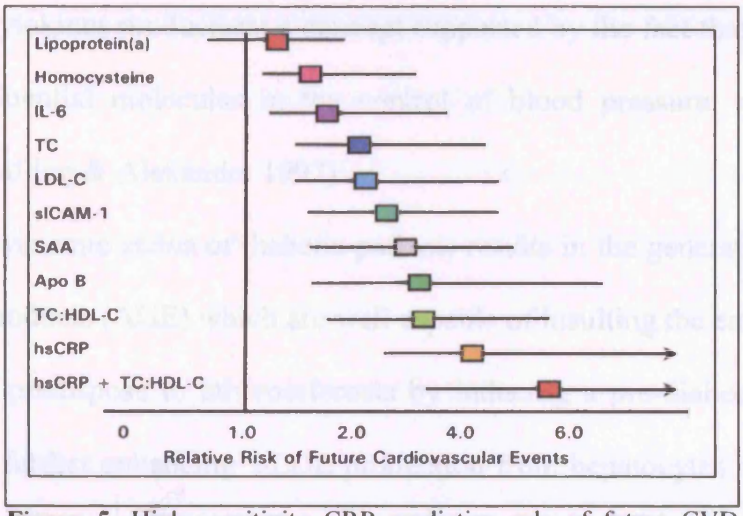


**Figure 5.** High sensitivity CRP predictive role of future CVD events compared with other traditional cardiovascular risk factors. From epidemiological evidence CRP predictive role for future CVD events was greater than other novel inflammatory biomarkers (IL-6, sICAM-1) as well as of traditional cardiovascular risk factors (LDL-cholesterol). Maximum prediction was obtained combining informations from CRP and lipid markers.
Adapted from (Bassuk et al. 2004)

reduction in serum CRP concentrations (Ridker et al. 1998; Ridker et al. 1999).

The exact mechanisms behind the link of inflammation and cardiovascular events, however, are still under careful consideration.

It might well be that the APR observed in so many populations considered healthy was the consequence and not the cause of a latent atherosclerotic process. Many investigations have demonstrated a clear significant local (atheroma) production of inflammatory cytokines (Berliner et al. 1995; Libby et al. 2002). Evidence supporting a series of possible other triggers however has been accumulating over the past decade:

i) LDL bound to proteoglycans can undergo oxidative modification within the intima wall and are capable of inducing expression of cell adhesion molecules as well as chemokines and pro-inflammatory cytokines (Berliner et al. 1997).

ii) Increased VLDL serum levels can be oxidized in a similar manner or the hypothesis is that they can themselves stimulate a vascular inflammatory reaction (Dichtl et al. 1999).

iii) Hypertension can influence intima homeostasis and induce lipid oxidation and pro-inflammatory cytokines production; a concept supported by the fact that Angiotensin II, one of the influential molecules in the control of blood pressure, has shown such activities (Griendling & Alexander 1997).

iv) The hyperglycaemic status of diabetic patients results in the generation of advanced glycation end products (AGE) which are well capable of insulting the endothelial cells.

v) Obesity can predispose to atherosclerosis by inducing a pre-diabetic state (insulin resistance) and further enhancing VLDL production from hepatocytes due to increased circulating free fatty acids (Yudkin et al. 1999; Yudkin et al. 2004).

A possible aetiologic activity of CRP itself has been recently proposed due to the protein capacity of enhancing each step of the atherosclerotic process within the intimal wall (Libby et al. 2002). Further evidence of its pivotal role in atherogenesis comes from the discovery of the involvement of acute systemic inflammation with acute

cardiovascular events. As previously mentioned APR is common after tissue ischemia events (Pepys & Hirschfield 2001). Serum concentrations of IL-6 and CRP are elevated after an acute coronary event. These inflammatory markers correlate with the rate of hospitalization and adverse prognosis (Liuzzo et al. 1999; Biasucci et al. 1999) and may represent not just the consequence of tissue necrosis but also the primary inducer of the vascular instability. Patients with unstable angina without myocardial necrosis but who present later with recurrent episodes of infarction and complications have consistently higher inflammatory markers levels (Liuzzo et al. 1999; Biasucci et al. 1999).

Finally, the infectious hypothesis has gathered increasing consensus in the last decade. After reviewing the attributable cause of all cardiovascular events, 30-40 % of cases cannot be explained by the known cardiovascular risk factors (Fong 2000; Kiechl et al. 2001; Kol & Santini 2004; Leinonen & Saikku 2002).

A series of studies has associated chronic and acute infections with cardiovascular diseases. Starting with childhood acute infections might increase the risk of atherosclerosis (Leinonen & Saikku 2002). Furthermore preceding the events of an acute myocardial infarction an influenza-like syndrome has been reported in the literature many times (Leinonen & Saikku 2002). Acute infections can impair endothelial function and therefore pose a threat to general health (Bhagat & Vallance 1997; Hingorani et al. 2000). Sero-epidemological studies have been reported linking CMV, HSV, and *C. pneumoniae* to atherosclerosis (Leinonen & Saikku 2002; Muhlestein & Anderson 2003; Shah 2002). The evidence produced on the infectious aetiology of atherogenesis is based upon the discovery of such pathogens in atherosclerotic plaques (*C. pneumoniae* in particular) and on animal experimental models where the onset and progression of atherosclerosis was enhanced by the presence of the specific pathogen as well as in vitro research showing potential atherogenic activity of such infectious agents. Possible mechanistic explanations

include: i) pathogens may induce inflammatory changes within the atheroma, ii) some infectious agents (*C. pneumoniae*) in the presence of LDL can accelerate macrophage transformation into foam cells, iii) an immune response towards the pathogens or their product might cross-react with host components [e.g. bacterial Heat Shock Proteins (HSP)], iv) chronic infections may produce a low-grade inflammatory systemic status, assessed by increased CRP levels and thus contribute to the atherosclerotic process by inducing inflammatory responses, v) finally the presence of not just one but several infectious agents (pathogen burden) throughout the individual lifespan might have an additive effect on the individual inflammatory burden and therefore predispose or influence atherosclerosis (Leinonen & Saikku 2002).

Chronic infections furthermore can interact with other cardiovascular disease risk factors such as age, smoking, gender and diet (Leinonen & Saikku 2002). In particular several pathogens have shown the ability to produces changes in lipid metabolism. Infections can cause a state of hyperlipidaemia due to both a decreased clearance and/or increased hepatic lipoprotein production. Pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) can mediate changes in lipid metabolism (Yudkin et al. 2000). Bacterial toxins (LPS) can also induce changes in cholesterol concentrations (reduced HDL and increased LDL) or target glucose metabolism and produce a state of IR. Among other emerging cardiovascular risk factors, fibrinogen concentrations can be influenced by chronic infections inducing a systemic pro-coagulant state that ultimately may represent a crucial pathogenetic link between infections and the atherosclerotic process (Lowe 2001; Rader 2000).

### 1.2.4 METABOLIC SYNDROME

Metabolic syndrome is defined as the presence of at least two of the following factors: abdominal obesity, atherosclerosis, IR and hyperinsulinaemia, hyperlipidaemia (elevated levels of triglycerides and reduced HDL-cholesterol), essential hypertension,

type-2 diabetes and coronary heart disease (Fernandez-Real & Ricart 2003; Yudkin et al. 2004). Risk factors for these disorders seem to share a common inflammatory or genetic basis although the precise pathogenetic mechanisms are still unknown. A low-grade state of systemic inflammation defined by raised concentrations in serum of inflammatory markers (especially IL-6 and CRP) has been demonstrated both before and after the onset of this syndrome and might represent the missing link among them. The trigger of this systemic inflammation however is still unknown. Obesity or hyperglycaemia can both induce an increased production of IL-6 from adipocytes (Mohamed-Ali et al. 2001; Yudkin et al. 1999; Yudkin et al. 2000; Yudkin et al. 2004) or endothelial cells and macrophages respectively (Chae et al. 2000; Morohoshi et al. 1996). Dyslipidaemia, with increased oxidation of LDL together with a reduced availability of NO, may then develop and explain why cardiovascular diseases are so common in individuals suffering from metabolic syndrome.

A chronic infection could well be the cause of the association between acute-phase markers, insulin resistance and atherosclerosis. In this unifying view a reduced insulin action is the consequence of the host response to an external insult.

The inter-individual variability to mount an acute phase response is also genetically determined (Baumann et al. 1984; Castell et al. 1990; Kushner 1988; Moshage et al. 1988). Polymorphisms in pro-inflammatory genes responsible for hyper-inflammatory phenotypes could be the consequence of the selective pressure of infectious diseases (Tabrizi et al. 2001; Waterer & Wunderink 2003). It may be that these genetic variants are more beneficial in eradicating the infectious insults but on the other-side the continuous exaggerated acute-phase response produced may contribute to insulin resistance. Further investigations, however, are needed to clarify the still unknown and complex gene and environment interactions.

## 1.3 APR AND PERIODONTAL DISEASE

### 1.3.1 PERIODONTAL INFECTION

#### DEFINITION

Periodontitis is a chronic infection which differs from gingivitis by loss of the connective tissue attachment to the teeth and resorption of the alveolar bone (Williams 1990). The process is initiated by the accumulation of a dental plaque biofilm on the tooth surface near the gingival margin (Page 1991; Page & Kornman 1997). Moderate forms of periodontal disease are thought to be highly prevalent (40-50% of the adult population) whereas severe forms affect only a minority of adults (10%) (Papapanou 1996; Papapanou 1999).

#### PATHOBIOLOGY

The aetiology and pathogenesis of periodontitis are still incompletely understood even though microbial plaque is considered the crucial factor in the initiation of the gingival inflammation (De Nardin 2001). Supra- and subgingival plaque, once colonized by specific gram negative microbes (e.g. *Prophyromonas gingivalis, Tannerella forsythensis, Actinobacillus actinomycetemcomitans, Campylobacter rectus, Prevotella intermedia* and some spirochetes) directly elicits a defensive inflammatory response within the gingival tissue (Moore & Moore 1994; Socransky 1984). The individual host response to the bacterial insult represents therefore the other essential component in the pathogenesis of periodontitis.

Bacteria or their toxic products (LPS) within the gingival sulcus may gain access to the gingival tissue and recruit a local inflammatory infiltrate characterized by neutrophils, lymphocytes and macrophages. Local macrophage activation results in production of pro-inflammatory cytokines such as IL-1$\alpha$ and $\beta$, TNF-$\alpha$ and IL-6 in order to contain/clear the insult (Ebersole et al. 1993; Kinane et al. 1991; Lamster & Novak 1992; Page 1991; Page & Kornman 1997). The pathognomonic feature of periodontitis,

i.e. attachment loss, occurs as the result of matrix metalloproteinase activation in response to cell stimulation by pro-inflammatory cytokines (Birkedal-Hansen 1993; Page 1991). IL-1 in particular has been involved in bone resorption and amplification of the inflammatory infiltrate (Offenbacher 1996). IL-6 is also involved in the pathogenesis of periodontitis and is highly represented in inflamed periodontal tissues (Irwin & Myrillas 1998).

Periodontal infection also causes a specific local immune response characterized by increased local infiltration of T helper and suppressor lymphocytes together with raised circulating levels of IgG antibodies (Ebersole et al. 1987). Due to possible sequence homology between human and bacterial components (e.g. heat shock proteins, HSP), immuno-complexes formation and deposition both in the local vasculature and connective tissue may also induce a self-perpetuating local autoimmune reaction independent of the presence of the bacterial trigger (Offenbacher 1996; Page 2002).

Because of the loss of connective tissue attachment secondary to the activation of the host matrix-metalloproteinases, there is a pathologic migration of the gingival epithelial attachment along the root surface that may reach the apex of the tooth rendering tooth loss inevitable. The deepened gingival sulcus (periodontal pocket) represents the pathognomonic lesion of periodontitis. A new local environment is produced and bacterial colonization increases. Micro-colonies of oral bacteria deposit on soft and hard tissues being selected by the new anaerobic environment (Socransky et al. 2002; Socransky & Haffajee 2002). Based on the degree of the inflammatory reaction, an ulceration of the epithelium lining the pocket may also facilitate pathogens' invasion of the periodontal tissues (De Nardin 2001; Wank et al. 1976). Once in the gingival tissues some bacteria may well find their way into the bloodstream especially during tissue stressing events such as chewing, brushing or dental treatment (Bein et al. 2003; Bender 1995; Daly et al. 1997; Daly et al. 2001; Lofthus et al. 1991; McLaughlin et al. 1996;

Ramli 1998; Waki et al. 1990; Wampole et al. 1978). Despite their limited quantity and the rapid host reaction in clearing the insult, little is known about the potential harmful effect that such ephemerous bacteraemia can exert on systemic health.

The clinical course of periodontitis is characterized by alternating phases of active tissue destruction and phases of quiescence (Offenbacher 1996). Its insidious onset may not be evident to the individual affected who is often unaware of any symptoms until the later stages of the disease. Consequently the disease may remain untreated and progress for several months or even years. Clinical manifestation (localization of periodontal lesions), ethnic background and the presence of specific bacteria (*A. actynomycetemcomitans*) however have been associated with more severe or aggressive forms of periodontitis. High variability in the individual local inflammatory response to the same triggering bacteria has also been reported (Kornman 1999). Different genetic background seems to be the crucial determinant of such variability in some populations (Kornman et al. 1997). Common polymorphisms in inflammatory cytokine genes, for example, have been associated with more severe forms of periodontal infections (Kornman et al. 1997). Hyper-responding individuals or subjects with deficiencies in immune function (leucocytes PMN activity) also manifest with clinically more advanced disease (Genco 1996).

Periodontal treatment relies upon the elimination of the local dental plaque biofilm by mechanical instrumentation (sub-gingival) usually performed with local anaesthetic using hand and/or machine-driven (ultrasonic) instruments. Significant improvement of periodontal status occurs after combined therapy of oral hygiene instruction and thorough supra- and sub-gingival scaling and root planing in patients with periodontal infections. Local periodontal inflammation subsides with disappearance of the gingival swelling and bleeding. Recession of the gingival margin often occurs. Extractions of

teeth ultimately represent the last stage of periodontal treatment often because of little connective tissue attachment and alveolar bone remaining.

### 1.3.2 Systemic Implications of Periodontal Disease

The concept that oral infections might influence systemic health is not new and has always been one of great interest. The term "oral sepsis", later called "focal infection", circulated among physicians at the beginning of last century (O'Reilly & Claffey 2000). At that time it was postulated that distant "foci" of tissue infections (e.g. complicated carious lesions, advanced periodontitis) might be harmful for other body areas and therefore a tremendous effort in removing them (indiscriminate tooth extractions) developed (O'Reilly & Claffey 2000). With time and new clinical evidence the focal infection theory collapsed, since such an approach proved not beneficial in treating serious systemic diseases (rheumatism, arthritis, kidney diseases) (O'Reilly & Claffey 2000).

The focal hypothesis has seen new light in the past decade since a series of research investigations have associated periodontitis with various systemic diseases (cardiovascular disease, diabetes, pulmonary infections and adverse pregnancy outcomes).

### 1.3.3 Diabetes

Diabetes mellitus and periodontitis have been associated for many years (Taylor et al. 1996; Taylor 2001; Zambon et al. 1988). Diabetic patients show a significantly higher likelihood of developing periodontal diseases than non diabetics and many physicians are aware of this relationship. For several years though this relationship has been examined only in one direction (uncontrolled or poorly controlled diabetes increases risk of periodontitis). Some reports however have shown how periodontal therapy may affect glycaemic control (Grossi et al. 1997; Grossi & Genco 1998; Taylor et al. 1996)

raising a new hypothetical association: periodontal chronic infections may increase the risk of developing type-2 diabetes or may well aggravate the metabolic homeostasis in diabetic patients.

It is widely recognized that chronic infections may produce endocrine-metabolic changes resulting in poorer glycaemic control and insulin resistance (see section 1.2.1). Independent epidemiologic surveys moreover have demonstrated that the prevalence of type-2 diabetes and its complication are higher in subjects with periodontitis when compared with unaffected populations (Grossi & Genco 1998). A bidirectional relationship between periodontitis and in particular type-2 diabetes seems therefore significant.

Diabetes in adulthood is often preceded by a status of insulin resistance, elsewhere called glucose intolerance (Fernandez-Real & Ricart 2003). Diabetic patients might be suffering from a defect of the innate immune system perhaps triggered by a chronic low-grade inflammatory systemic state (Fernandez-Real & Ricart 2003). In spite of the fact that obesity represents the more studied among type-2 diabetes risk factors (e.g. age, diet, smoking), the common underlying pathogenetic mechanism of insulin resistance is often associated with systemic inflammation (Fernandez-Real & Ricart 2003). As already mentioned in section 1.2.1, systemic inflammation is influenced by insulin action at different levels and vice-versa. Insulin resistance syndrome may represent the consequence of repeated acute phase responses in predisposed individuals whenever they are challenged by external pathological insults (Fernandez-Real & Ricart 2003; Yudkin et al. 2004). Chronic infections such as periodontitis can trigger a systemic inflammatory response by different means: i) haematogenous dissemination of periodontal pathogens and ii) local excessive production of pro-inflammatory cytokines (TNF-α, IL-1 and 6) dumped into the bloodstream (Page 1998). Impaired glucose tolerance may therefore develop and precede the onset of type-2 diabetes. Recent

investigations have demonstrated a significant association between local periodontal infection, systemic markers of inflammation and glycaemic regulation in individual suffering from severe periodontitis (Saito et al. 2004).

### 1.3.4 ADVERSE PREGNANCY OUTCOMES

Preterm low birth-weight (PLBW) delivery (before 37$^{th}$ week of gestation and < 2500g of weight) represents a possible consequence of maternal young age, alcohol, drug, tobacco use and genetic background (Smith 2004). However a large body of clinical evidence suggests that infections (mainly of the genitor-urinary tract) can account for a significant number of prematurities through an inflammatory reaction involving the uterus and annexes (Smith 2004). Direct bacterial invasion of the feto-placental unit and an excessive production of pro-inflammatory mediators may affect gestational age and predispose to PBLW (Madianos et al. 2001; Offenbacher et al. 1998b; Offenbacher et al. 2001; Offenbacher et al. 1996). Interest on the possible role of not just local but also distant tissue infections has therefore increased.

In this respect, an investigation suggested that maternal periodontal infections can significantly increase the risk (nearly 8 times) of PBLW delivery and intra-uterine growth restriction (Offenbacher et al. 1996). A large prospective trial (1300 patients) confirmed that maternal periodontitis was an independent risk factor for PBLW (Jeffcoat et al. 2001) while a more recent case-control study failed to support this conclusion (Davenport et al. 2002).

Some evidence however has been produced investigating the effect of periodontal therapy during pregnancy. A cohort study led to the conclusion that achieving periodontal health during pregnancy would have a beneficial effect on gestational age and infant birth weight (Mitchell-Lewis et al. 2001). Similar results were reported from a randomized intervention trial showing a significant difference between the incidence

of prematurity among women who were treated for periodontal disease and those who were not ( 10 vs 1.8 % incidence of gestational age < 37 weeks respectively) (Lopez et al. 2002).

Although local infections (such as vaginosis), maternal age, smoking alcohol still represent the leading cause, periodontal experimental models (hamster) demonstrated a significant association between presence of periodontal pathogens, experimental periodontitis and feto-placental toxicity including foetal weight (Collins et al. 1994a; Collins et al. 1994b).

Periodontal infections may also cause other adverse pregnancy events such as pre-eclampsia, which commonly results in maternal hypertension, proteinuria and affects maternal mortality and morbidity (Boggess et al. 2003). In a large cohort of pregnant women when periodontitis was present and showed progression, the risk of pre-eclampsia increased significantly and independent of other recognized risk factors (maternal age, smoking etc) (Boggess et al. 2003). Investigators concluded that maternal periodontal disease would represent a foetal stressor acting directly through haematogenous dissemination of pathogens (or their toxins) and produce placental damage or indirectly by a stimulated maternal inflammatory-immune reaction (Boggess et al. 2003). An increased concentration of pro-inflammatory cytokines and mediators has been reported both in maternal and foetal circulation (Offenbacher et al. 2001).

### 1.3.5 CARDIOVASCULAR DISEASES

Many studies in the 1990s have linked dental infections with increased risk of CVD (Table 2 ). Since the first case-controlled studies were published in 1989, significant positive associations between various cardiovascular diseases (myocardial infarction, hospitalization, cardiac sudden death and peripheral vascular disease) and measures of oral health (alveolar bone loss, CPTIN, Russell Index, etc.) have been reported. Two

recent meta-analysis indicated that periodontitis (defined by all measures reported in literature) resulted in a small significant increased risk of developing cardiovascular events (OR=1.19 (95% CI, 1.08 1.32) (Janket et al. 2003), (OR=1.13, 95%CI 1.01-1.27) (Khader et al. 2004) independent of age, gender, smoking and other confounders.

**Table 2** Epidemiological evidence of periodontal-CVD "connection"

| STUDY | DESIGN | ENDPOINT | RATIO |
|---|---|---|---|
| (Mattila et al. 1989) | Case/control | Fatal MI or new MI | 1.3 |
| (Mattila 1993) | Case/control | Fatal MI or new MI | 1.4 |
| (Mattila et al. 1995) | Follow up | Fatal MI or new MI | 1.2 |
| (DeStefano et al. 1993) | Longitudinal | Admission to hospital/ fatal CHD | 1.7 |
| (Beck et al. 1996) | Longitudinal | New or fatal CHD, Stroke | 1.5,1.9, 2.8 |
| (Joshipura et al. 1996) | Longitudinal | Fatal/non Fatal MI CSD | 1.0 |
| (Grau et al. 1997) | Case/control | Non fatal ischemic Stroke | 2.6 |
| (Loesche et al. 1998) | Cross-sectional | CHD | 2.6 |
| (Morrison et al. 1999) | Retrospective | Fatal CHD Stroke | 3.4 |
| (Arbes, Jr. et al. 1999) | Cross-sectional | History of heart attack | 2.2* |
| (Wu et al. 2000a) | Longitudinal | Incident non hemorrhagic Stroke | 2.1 |
| (Hujoel et al. 2000) | Longitudinal | Admission to hospital/ fatal CHD, Revascularization | 1.1 |
| (Howell et al. 2001) | Longitudinal | Fatal CHD, non fatal MI, stroke | 1.1 |
| (Jansson et al. 2001) | Longitudinal | Fatal CVD | 2.7 |
| (Buhlin et al. 2002) | Cross-sectional | Self-reported CVD and Stroke | 1.6 |
| (Joshipura et al. 2003) | Longitudinal | Ischemic Stoke | 1.3 |

* Crude OR for individuals with 1/3 of the sites showing CAL ≥ 3mm

The National Health and Nutrition Epidemiologic follow-up Study (NHANES) (DeStefano et al. 1993) was one of the large cohorts analysed. Different periodontal research groups however reported contrasting conclusions. Although the first analysis demonstrated a strong association of both, periodontal disease and poor oral hygiene, with total mortality for CHD, particularly among young and middle-aged men (aged 25-49 years) a different analysis from a database with longer follow-up of the same cohort showed no association (Hujoel et al. 2000). A larger prospective study (Joshipura et al. 1996) involving 44119 male health professionals who self reported their oral health status led to the same conclusions. On the other hand a strong association has been

repeatedly found between periodontal disease and tooth loss with ischemic stroke (Grau et al. 1997; Joshipura et al. 2003).

The controversial association between periodontitis and atherosclerosis perhaps is strongly confounded by many common risk factors that the two inflammatory diseases share (age, male gender, diabetes mellitus, host susceptibility, stress, smoking poor socio-economic status) (Genco et al. 2002; Genco & Slots 1984; Kinane 1998a; Kinane 1998b). Certainly among them the role of cigarette smoking perhaps represents the strongest confounder (Hyman et al. 2002).

Nevertheless, biological plausibility of this association is supported by many lines of research: i) periodontitis together with other chronic infections (*C. pneumoniae, H pilory*, CMV) have been associated with increased odds of future cardiovascular events; the relative risk observed for the various infections was of similar magnitude (Danesh 1999), ii) experimental animal and *in vitro* evidence have been produced about the potential role of periodontal pathogens on the atherosclerotic lipid deposition process (Jain et al. 2003; Lalla et al. 2003; Li et al. 2002) and thrombo-embolic complications (Herzberg & Weyer 1998), iii) periodontal pathogens (*P.gingivalis*) have been localized within atherosclerotic plaque in analogy with *C. pneumoniae* (Haraszthy et al. 2000), iv) *P. gingivalis* is able to invade vascular tissues (Dorn et al. 2000) and facilitate macrophage transition to foam-cells (Miyakawa et al. 2004; Qi et al. 2003), v) finally, the presence of several intraoral pathogens (pathogen burden) has been associated with an increased host inflammatory response measured by elevated serum concentrations of acute phase markers (Noack et al. 2001).

Despite the number of reports that show a positive association between periodontal infections and cardiovascular events, these investigations do not prove any causal relationship between those two diseases. A recent systematic review on the matter concluded that although moderate levels of evidence have been produced on this

association, additional larger epidemiological and interventional studies are necessary (Scannapieco et al. 2003).

### 1.3.6 Systemic Host Response

Pro-inflammatory cytokines are significantly elevated within the gingival tissue during the active phase of periodontitis (Offenbacher et al. 1981). Increased concentrations of many inflammatory mediators have been detected in the gingival crevicular fluid (an exudate originating at the dento-gingival junction) presumably due to the local bacterial challenge and the local host defence activity (Offenbacher et al. 1981). Bacteria or their toxic products may easily gain access to the circulatory system. Brief episodes of bacteraemia have been detected after normal activity such as chewing, brushing or more clearly as a consequence of periodontal therapy (Bender 1995; Carroll & Sebor 1980; Coulter et al. 1990; Daly et al. 2001). Individuals with severe generalized forms of periodontitis are at greater risk of showing such episodes, even though the systemic host response overall neutralizes bacterial activity efficiently and without showing clinical signs. A serum antibody response is therefore observed and in particular an increase in specific IgG against common periodontal pathogens has been reported after successful standard periodontal therapy (Ebersole et al. 1987). Similar to bacterial dissemination, an excessive local production of pro-inflammatory cytokines can be dumped into the blood stream and trigger a systemic acute-phase response.

In the last 5-7 years many reports focused on the possible link between severe periodontitis and systemic acute phase response. Consequently a renewed interest on the possible harmful role of the systemic host reaction to periodontal chronic infections has been produced. In table 3 are listed some of the investigations that reported increased concentrations of acute phase reactants in serum of individuals suffering from severe periodontitis as opposed to healthy subjects. Kweider and co-workers found in a population of periodontitis patients that acute phase markers, such as fibrinogen and

WBC, were elevated in comparison with unaffected individuals (Kweider et al. 1993). Since that investigation few scattered reports have confirmed such findings in small case control studies. Periodontitis patients have higher CRP and IL-6 levels when compared with matched periodontally healthy populations (Amar et al. 2003; Ebersole et al. 1997; Glurich et al. 2002; Loos et al. 2000; Noack et al. 2001). This perturbation of the physiological homeostasis is also accompanied by a lower number of erythrocytes and haemoglobin concentrations (Hutter et al. 2001), higher values of haptoglobin, moderate leukocytosis (Fredriksson et al. 2002) and increased cholesterol, LDL and glucose levels (Katz et al. 2002; Losche et al. 2000; Wu et al. 2000b).

Some reports concluded also that the extent and severity of periodontitis was positively associated with greater systemic acute phase response. The acute phase response observed in periodontal patients was also associated with the presence of local periodontal pathogens (Noack et al. 2001) and was independent from other systemic chronic infections (seropositivity to *C. pneumoniae, H pilory*, CMV) (Loos et al. 2000). Due to the limited number of individuals, however, the main criticism raised to these investigations was the inability to efficiently account for a series of possible confounders (age, smoking, diet, diabetes) that may well affect the acute phase response as mentioned earlier. Nevertheless recent larger cohort longitudinal studies provided new evidence that periodontal disease is associated with systemic and cardiovascular risk factors including acute phase proteins (Joshipura et al. 2004; Slade et al. 2000; Slade et al. 2003; Wu et al. 2000b). Using data from the NHANES III, the ARIC, Health Professional Study populations, investigators have found that individuals with periodontitis have increased systemic levels of CRP, Fibrinogen, t-PA and LDL cholesterol. These differences were also significant when the analyses accounted for a series of possible confounding factors (age, sex, smoking, socioeconomic status, diabetes, body mass index, alcohol use).

**Table 3** Periodontitis and APR

| STUDIES | DESIGN | SUBJECTS (CONTROLS) | HEALTHY | PERIODONTITIS |
|---|---|---|---|---|
| (Ebersole et al. 1997) | RCT | 40 (35) | 2.17± 0.41 | 9.12 ± 1.61 |
| (Fredriksson et al. 2002) | Case/Control | 83 (43) | 0 | 2 |
| (Loos et al. 2000) | Case/Control | 150 (43) | 0.90 | 1.45 |
| (Slade et al. 2000) | Prospective | 12949(2253) | 3.3 ± 0.1 | 4.5 ± 0.3 |
| (Noack et al. 2001) | Case/Control | 174 (65) | 1.70 ± 1.91 | 4.06 ± 5.55 |
| (Glurich et al. 2002) | Case Control | 80(44) | 1.68 ± 1.42 | 2.40 ± 1.89 |
| (Amar et al. 2003) | Case Control | 55 (29) | 1.0 ± 1.0 | 2.3 ± 2.3 |
| (Slade et al. 2003) | Prospective | 4504(4250) | 5.8 ± 0.13 | 7.6 ± 0.65 |
| (Joshipura et al. 2004) | Cross/Sectional | 468 (377) | 1.8 ± 3.0 | 2.2 ± 2.5 |

Until now, only limited evidence has been reported from intervention studies aimed at establishing the nature of the association. Conflicting results have been observed after small pilot intervention studies. In some trials, the results of a negative association have been questioned because of the limited effect of the delivered periodontal therapy. In others high variability in terms of changes in acute phase markers was observed after therapy (Christan et al. 2002; Ide et al. 2003; Iwamoto et al. 2003; Mattila et al. 2002).

# CHAPTER 2.

# EXPERIMENTAL HYPOTHESIS

## 2.1. EXPERIMENTAL HYPOTHESIS

Periodontitis has been associated with a series of systemic diseases (atherosclerosis, cardiovascular events, adverse pregnancy outcomes and metabolic control of type-2 diabetes). The underlying pathogenetic mechanism(s) might lie in the host APR to periodontitis with its local inflammatory and infectious burden. Results of the association studies, the chronological consistency of the exposure, the biological plausibility, and data from animal models support the existence of a causal link between severe periodontal infections and a systemic inflammatory response of magnitude similar to that considered predictive of future development of systemic diseases such as: atherosclerosis, hypertension, or adverse pregnancy outcomes. The periodontal local infectious stimulus and the host inflammatory response associated with it might generate a systemic APR. Among the possible mechanisms through which periodontitis might influence systemic homeostasis we hypothesise: i) an excessive local production of pro-inflammatory cytokines (IL-1, TNF-α) which triggers systemic production of IL-6 and therefore induces a systemic APR; ii) periodontal pathogenic bacteria (*Pg,Tf,Aa*) or their toxic products (i.e. LPS) might invade local tissue and directly stimulate IL-6 production or can directly disseminate into the bloodstread (ephemerous bacteraemias)

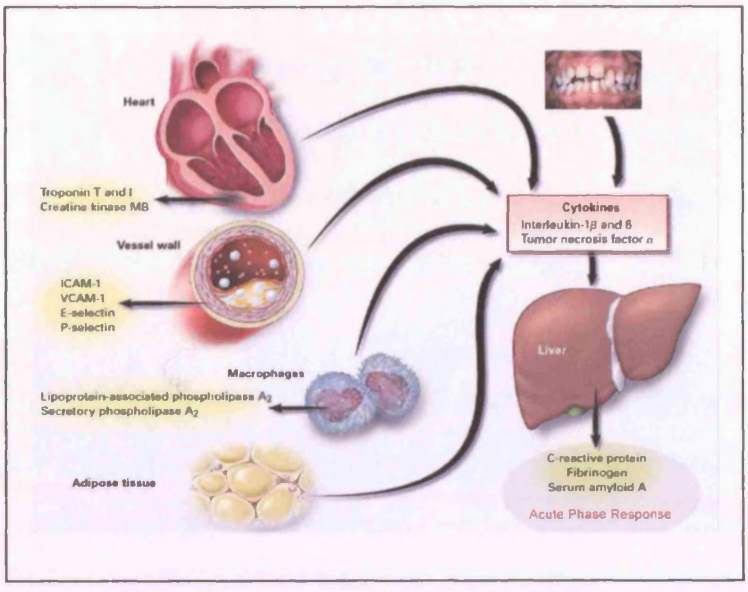


**Figure 6.** Possible triggers of APR
Periodontitis may represent a moderate stimulus for a systemic acute phase response. Recognized inflammatory stimuli are obesity, vascular or myocardial tissue damage. Adapted from (Rader 2000)

producing a systemic APR; iii) a combination of the two.

A chronic systemic inflammatory state can predispose individuals to future serious events (CVD, diabetes, stroke, PBLW) (see chapter 1).

The experimental hypothesis of this work was that periodontitis contributes to the overall inflammatory burden of the subject.

We selected to quantify in serum and blood both crude and sensitive markers that we assumed would represent a reliable method of assessing the individual inflammatory burden (WBC, CRP, IL-6). If the association between systemic inflammation and the local periodontal infection were causal in nature, successful treatment of periodontitis would lead to a significant decrease in these inflammatory markers. In order to assess the hypothesis we selected patients with severe and generalized forms of periodontitis based on previous evidence indicating a dose response between exposure and cardiovascular events in an association study (Slade et al. 2000; Slade et al. 2003; Wu et al. 2000b).

The influence of individual genetic susceptibility (polymorphisms in genes encoding cytokines) and of well recognized individual confounding factors (age, gender, ethnicity, body mass index, cigarette smoking) on patient response to periodontal therapy was also explored.

## 2.2 SPECIFIC AIMS

- to assess, using a multi-level analysis approach, the overall efficacy and relative contribution of patient, tooth and site-associated factors in determining the clinical outcomes of standard periodontal therapy consisting of sub-gingival debridement performed with a machine-driven instrument. (**Chapter 4**)
- to assess whether the degree of individual response to periodontal treatment was associated with changes in serological markers of systemic inflammation (CRP, IL-6) in a cohort of otherwise healthy individuals (**Chapter 4**)
- to preliminary explore the effects of periodontal therapy on changes in the recently defined CRP-associated CVD risk in a group of otherwise healthy individuals. (**Chapter 4**)
- to investigate the possible association between specific cytokine polymorphisms and the systemic inflammatory response (estimated as serum CRP and IL-6) in individuals suffering from severe generalized periodontitis. (**Chapter 4**)
- to describe the changes in systemic inflammatory parameters consequent to an intensive periodontal treatment regimen, and to determine the kinetics of changes in inflammatory markers and mediators (CRP, IL-6, IL-1Ra) in the early days following the delivery of periodontal treatment, an event that has been associated with bacteraemia. (**Chapter 5**)
- to evaluate the effect of a novel polymorphism (+1444C>T ) on CRP acute release after intensive periodontal therapy and its dependence from the IL-6/–174G>C polymorphism and standard cardiovascular (CV) risk factors. (**Chapter 5**)
- to test in a 3-arm randomized, controlled intervention trial the short term effects of different regimens of periodontal therapy on the systemic inflammatory status of medically healthy individuals suffering from severe, generalized periodontitis. (**Chapter 6**)

- to investigate in a randomized controlled trial whether different periodontal therapy regimens (standard vs intensive) were associated with a better control of the local and systemic inflammatory response. (**Chapter 7**)

## 2.3 EXPERIMENTAL PLAN

Five interventional studies (three cohort studies and two randomized controlled clinical trials, **Chapter 4, 5, 6, 7**) were designed to ascertain whether the control of periodontal infection by different treatment modalities would affect the individual systemic inflammatory burden.

**Chapter 4** describes the preliminary association between systemic inflammation and severe periodontitis in a cohort of 94 otherwise healthy individuals. We assessed the correlation between changes in serum levels of acute phase markers and individual genetic susceptibility, severity and extent of periodontal infection, and outcomes of standard periodontal therapy. (The candidate acted as periodontal examiner and collected biological samples. He performed serum quantification of IL-6).

With the second small intervention trial (**chapter 5**) we wanted to explore the acute phase response to a moderate inflammatory stimulus (represented by the delivery of periodontal therapy). This study also allowed the description of periodontal therapy as a novel non drug-induced model to study systemic inflammation (The candidate acted as therapist and performed serum quantification of IL-6, IL-1Ra).

We then performed a larger cohort study to confirm the data obtained from this trial and in particular we addressed the question of whether or not genetic variation (polymorphisms) may contribute to the inter-individual variation in response. (The candidate acted as therapist and performed IL-6 quantification).

We then performed a randomized controlled pilot intervention trial (**chapter 6**) to evaluate the short term impact of two different periodontal treatment regimens (sub-gingival machine-driven instrumentation with or without local administration of an antimicrobial) compared to supragingival scaling and polishing alone on the control of the local and systemic inflammatory response associated with severe chronic periodontitis. (The candidate acted as therapist and performed IL-6 quantification).

The second randomized controlled trial (**chapter 7**) was designed to test the hypothesis of a causal association between severe periodontitis and systemic inflammation. Changes in inflammatory markers were monitored for 6 months after treating

systemically healthy individuals affected by severe generalized periodontitis with either a standard periodontal treatment regimen or an intensive one. (The candidate acted as therapist and performed IL-6 quantification).

# CHAPTER 3.

# GENERAL MATERIAL AND METHODS

## 3.1 STUDY CONDUCT

### 3.1.1 LOGISTICS

All clinical intervention trials presented in this thesis were performed at the Eastman Clinical Investigation Centre (ECIC) of the Eastman Dental Hospital and Institute (EDI), University College London (UCL). The staff of the centre have been trained and dedicated only to clinical research trials. Each clinical trial taking place at the centre was conducted in accordance with the declaration of Helsinki on experimentation involving human subjects. Study protocols were peer reviewed and approved by the joint local Ethical Committee of the Eastman Dental Hospital and UCL Hospitals. Each trial was also registered with the Research and Development Directorate of the UCL Hospital Trust.

### 3.1.2 CLINICAL TRIAL DESIGN AND ORGANIZATION

The screening phase of possible candidates for the trials took place within the new-patients clinics of the department of periodontology. Individuals referred for periodontal therapy to the hospital were first examined by the department consultants. A general clinical periodontal examination was performed. If the subjects fulfilled the specific inclusion/exclusion criteria for the specific trial (Table 4) the information was presented by the attending consultant with the assistance of an information sheet (See Appendix 1 for example).

The study was presented verbally to the patient by the consultant presenting the benefits and risks of participation. The patient was advised that the choice to participate was voluntary and their decision would not affect their future care in the hospital in any way. The patient was given the opportunity to ask questions. The trial information sheet was given to the patient to take home. They were advised to read it and be sure that they understood it fully. Following a minimum period of one week, the clinical research

coordinator contacted the patient to ask if they had any questions regarding the information sheet and if they had made a decision concerning participation. The consultant and members of the research unit were available to answer any possible query that the subjects asked. Based on a verbal agreement the baseline visit of the trial was scheduled and the patient, after signing a consent form (see Appendix 2 for example), was then re-examined.

**Table 4** Inclusion/Exclusion Criteria

| INCLUSION CRITERIA | EXCLUSION CRITERIA |
|---|---|
| Between the ages of 35 and 60 and in good general health. | Pregnant or lactating females. |
| Ability and willingness to follow study procedures and instructions. | Patients chronically treated (i.e., two weeks or more) with any vasoactive medication (e.g., anti-hypertensive), lipid lowering therapy or anti-inflammatory therapy (steroids, NSAID). |
| Must have read, understood and signed the Informed Consent. | Diagnosis of uncontrolled metabolic diseases including diabetes, kidney, liver, or cardiovascular diseases. |
| Individuals must have generalized, moderate to advanced chronic periodontitis as measured by the presence of ≥ 40 periodontal pockets with bleeding on probing. | Use of systemic antibiotics in the preceding 3 month or patients who require antibiotic pre-medication for the performance of periodontal examination or treatment. |
| | Patients suffering from chronic infectious diseases such as hepatitis B, HIV and TB. |
| Absence of other significant oral infections. | Patients who received a course of periodontal treatment within the last 6 months. |

A comprehensive clinical periodontal examination was then performed by the study examiner who confirmed that the inclusion criteria were met. All data were recorded on study forms with the help of nursing staff. The subject was formally included in the trial and assigned a study number. Collection of biological (dental plaque, blood) samples was performed by the examiner. At the end of the baseline visit the patient left with a schedule of all subsequent study dates according to the specific experimental design. A reasonable time allowance was given for each study visit depending upon the informations required. If it was planned, randomization was performed by the Clinical Trial Coordinator and treatment allocation was concealed using opaque envelopes. The study therapist opened the treatment assignment envelope only at the study treatment

visit and only after having completed treatment common to both treatment groups. Clinical examiners remained blind to treatment allocation for the entire length of the study and subsequent data analysis.

### 3.2 CLINICAL EXAMINATION DATA AND EXAMINER CALIBRATION

At the baseline visit, a full medical history interview was first performed to ascertain whether there were any risks for the individual or to verify the specific inclusion/exclusion criteria for participation to the study. A detailed intra-oral examination was then performed. Standard periodontal clinical parameters were recorded in full mouth fashion by a single calibrated examiner at six sites per tooth

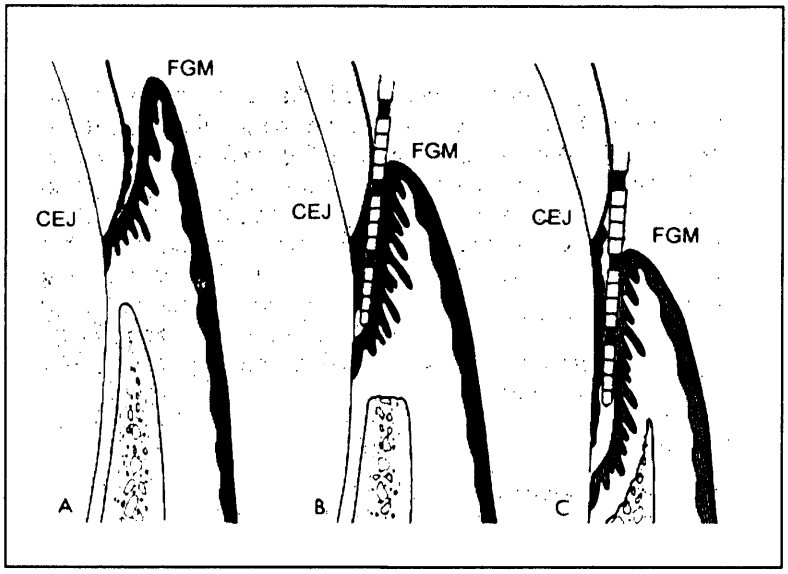


**Figure 7.** PPD and REC recording procedures
A: Healthy periodontium, B: Initial lesion, negative REC recording, C: Advanced lesion, positive REC recording

(disto-mid-mesio -buccal and -lingual) using a manual, UNC-15 periodontal probe (PCPUNC15, Hu-Friedy, Leimen, Germany) with light force. Full mouth probing pocket depths (PPD) from the free gingival margin to the deep end of periodontal pockets were recorded (Figure 7.). Whenever a clinical measure fell between two millimetre markings on the periodontal probe it was rounded up. At proximal areas the probe was placed against the contact surface and the tip was as close as possible to the interproximal space. With the periodontal probe inserted into the gingival pocket and

after calling the pocket depth reading, the examiner recorded the recession (REC) from the free gingival margin to the cemento-enamel junction (CEJ) by lifting the tip of the probe angled at 45° to the long axis of the tooth and seeking the tactile stop of the CEJ. Based upon the examiner discretion a different reference landmark (restoration margin, crown margin, incisal edge) was adopted and carried over at each examination visits whenever the location of the CEJ could not be detected. REC data were recorded as positive or negative numbers if the CEJ was exposed or covered by the free gingival margin respectively.

**Figure 8** Periodontal examination tray. Source: Hu-Friedy, Leimen, Germany

Clinical attachment levels (CAL) were calculated subsequently from the formula PPD plus REC (Machtei et al. 1993). A total of 10 non-study subjects were recruited and used for calibration of the examiner. These subjects had periodontal disease and the examiner recorded on two different occasions full mouth PPD and recessions at six sites per tooth (excluding third molars) using a manual, UNC-15 periodontal probe. The two sets of measurements were recorded at least 30 minutes apart. CAL was algebraically calculated from PPD and REC according to the above formula. Upon completion of all sets of measurements, intra-examiner repeatability for CAL measurement was assessed. The examiner was judged to be reproducible after meeting a percentage of agreement within ± 2 mm between repeated measurements of at least 98% for CAL.

Full mouth plaque scores (FMPS) were recorded at six sites per tooth as the percentages of surfaces which revealed the presence of plaque over the total number of surfaces examined (O'Leary et al. 1972). After recording PPD and REC the examiner waited up to 25 seconds to score the presence/absence of any signs of gingival bleeding. Bleeding upon probing (BoP) was recorded at six sites per tooth and full mouth scores were

calculated as above (FMBS) (Tonetti et al. 2002). Furcation involvement was graded as described by Hamp et al using a Nabers probe (PQ2N, Hu-Friedy, Leimen, Germany) while tooth mobility was assessed according to the Miller scale(Hamp et al. 1975). All clinical data were entered at the end of the trial in an electronic spreadsheet by research staff blind to the trial and error-proofed by the clinical trial coordinator.

Standard periapical radiographs were obtained for all patients from the Radiology service of the EDI using a long cone parallel technique and aiming devices (Updegrave 1951).

### 3.3 Microbiological Sample Collection

Samples of subgingival periodontal plaque were collected by the examiner at the baseline visits before recording any periodontal parameters and according to the study protocol. Sites were identified based on the clinical charting performed during the screening visit. Using a sterile curette (Gracey, Hu-Friedy, Leimen, Germany) the examiner collected subgingival plaque samples from the 4 deepest pockets, one in each quadrant (questionable teeth planned to be extracted, as defined in section 3.5.2, were not included) (Grossi et al. 1994; Socransky et al. 1994). At each sampling site supragingival plaque was gently removed with a scaler (SM13/14S6 Hu-Friedy, Leimen, Germany), the site isolated from saliva with cotton-wool rolls (Paul Hartmann, Lancashire, United Kingdom) and the curette inserted in the pocket. With a gentle stroke the curette was moved upwards from the bottom of the periodontal pocket to the free gingival margin. Samples were pooled and placed into 1 ml of reduced transport fluid (Syed & Loesche 1972)(see Appendix 3 for details). Specimens were immediately transported to the Department of Microbiology at the EDI for analysis. At each follow up visits scheduled, plaque samples were re-collected by the examiner from the same pockets irrespectively of the changes in probing pocket depth.

### 3.4 Blood Collection

Blood samples were collected from a single, clean venepuncture with minimal stasis from the antecubital fossa. Patients were seated in a comfortable reclined position and rested 5 minutes before the collection which took place before any dental procedure or examination was performed. Trained clinical staff located sites for collection (superficial veins of the upper limb: median cubital, basilic, cephalic) on the patient's arm with the help of a tourniquet (TourniClip Blue, Timesco, London, England)(Figure 9). A single needle-stick procedure was performed after disinfecting the skin (Swab saturated with Isopropyl Alcohol 70%, Seton Healthcare Group plc, Oldham, United Kingdom ) and the samples needed drawn using a 23 Gauge Butterfly Needle with a luer adaptor (Vacutainer® Systems, Blood Collection Set, ref 367263, Becton Dickinson, New Jersey, USA). The tourniquet was removed as soon as the blood appeared in the tube to minimize any local venous stress.

The first tube of blood was drawn for full blood count analysis in a 1 x 5ml K2EDTA spray dried plastic tube (Purple top, BD Vacutainer®, Becton Dickinson, New Jersey, USA) or discarded. The tube was labelled with patient information (name, hospital number, date and time of collection) with a water insoluble permanent marking pen with fine tip (Greiner Bio-One, Staedtler, Lumocolor) and sent for same day analysis to the Haematology Laboratories at the UCL Hospital Trust. A second sample for specific serological analysis was collected into 1 x 10ml glass tube with no additive (Red top, BD Vacutainer®, Becton Dickinson, New Jersey, USA) and an additional sample of blood (6ml K2EDTA plastic tube, Pink top,

**Figure 9** Blood collection sites.
Source: BD Becton Dickinson, New Jersey, USA

BD Vacutainer®, Becton Dickinson, New Jersey, USA) was collected for DNA extraction and subsequent genetic analysis. Tubes containing anticoagulant were inverted gently for 8 to 10 times after collection. All samples were then maintained upright, at room temperature (18-22°C) until centrifugation (the plain glass tube was allowed to clot for 30 minutes prior to centrifugation). Plain glass tubes were centrifuged at approximately 3800 rpm in a laboratory bench-top centrifuge (BR401, Denley, England) at 4°C for 10 minutes within 2 hours of collection. The supernatant serum was then removed, avoiding disturbance of the buffy coat, with a polypropylene transfer pipette (Brand Pasteur Plastic Pipette, Aldrich, Steinheim, Germany), and delivered into polycarbonate cryovials tubes in aliquots of 0.5ml (2.0 ml Cryogenic Vials, Cat. No. 5000-0020, Nalgene, Rochester, NY, USA). All cryovials were previously labelled using laser printed water and low-temperature resistant labels (L6008™, Heavy Duty Labels, Avery Dennison Corporation, Berkshire, United Kingdom). The aliquots of serum and the plastic K2EDTA pink tubes were frozen rapidly by transferring them to a -70°C freezer (U725, New Brunswick Scientific, Hertfordshire, United Kingdom), where they were maintained until processed. The samples were allowed to freeze rapidly, not placed in polystyrene racks.

Serum samples were thawed at room temperature and assayed in blind fashion in batches at the end of each clinical trial.

### 3.4.1 SERUM ANALYSIS

Quantification of serum inflammatory markers (CRP) was performed on an automated analyzer (Cobas Integra 700, Roche AG Diagnostics, Mannheim , Germany) at the Chemical Pathology Laboratories of the University College Hospital Trust.

The immuno-turbidimetric assay for serum quantification of CRP was standardized against CRM (Certified Reference Material) 470, 91/0619 RPPHS (Reference Preparation for Proteins in Human Serum) with a lower detection limit of 0.25 mg/L (Figure 10).

**Figure 10.** CRP Immunoassay Description
Source: Roche AG Diagnostics, Mannheim , Germany

Full blood counts were performed by means of automated haematology procedure (Automated Haematology Analyser SE-9000 RET/SYSMEX, Milton Keynes, United Kingdom).

### 3.4.2 CYTOKINES ASSAY

Cytokines assay (IL-6, IL-1Ra) procedures were performed on first time thawed serum samples by means of a high sensitivity quantitative two-stage sandwich enzyme immunoassay technique (Quantikine R&D Systems, Inc., Minneapolis, USA). Same patient samples collected at different time points were all analysed in a blind fashion on the same plate to minimize variability. Each plate consisted of a 96 well polystyrene microplate (12 strips of 8 wells); wells were coated with a mouse monoclonal antibody against the cytokine to be studied. We describe the laboratory procedures for quantification of IL-6 in serum providing informations according to the manufacturer instructions. IL-1Ra assay was performed in a similar fashion according to the manufacturer instructions.

### 3.4.2.1 REAGENTS PREPARATION

All reagents were brought to room temperature before use and prepared as follows:

**Wash buffer**: 100 mL of wash buffer concentrate were diluted into distilled water to prepare 1000 mL of wash buffer.

**Substrate Solution**: Solution was reconstituted from lyophilized substrate in 6 mL of substrate diluent.

**Amplifier Solution**: Solution was reconstituted from the lyophilized amplifier in 6 mL of amplifier diluent and thoroughly mixed.

**IL-6 Standard**: A stock solution of 10 pg/mL of standard was reconstituted with 5 mL of the calibrator diluent RD6-11. Standards were allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. 500 µL of the calibrator diluent RD6-11 concentrate were pipetted into each tube. The standard stock solution was used to produce a dilution series mixing each tube thoroughly before each transfer (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 pg/mL). Calibrator diluent served as the zero standard (0 pg/mL) (Figure 11).

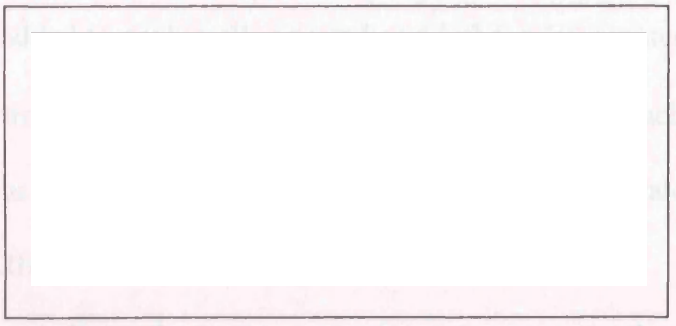

**Figure 11** IL-6 standards preparation.
Source: R&D Systems Inc., Minneapolis, USA

### 3.4.2.2 ASSAY PROCEDURE

After removing the microplate strip, 100 µL of assay diluent RD1-75 was added to each well. 100 µL of standard were added as duplicate into wells A-B 1 to 8. 100 µL of serum per well was afterward placed in duplicates in each consecutive well. Once the plate was full a cover adhesive strip was applied and incubated overnight at room temperature. The following morning the plate content was removed by inversion and

decantation. Any excess of liquid was carefully removed by rapping the plate inverted on a clean paper towel at least 5 times. Each well was then filled with 400 µL of wash buffer using a multi-channel pipette (Finnipipette Digital - Multichannel Runcorn, United Kingdom). The liquid was then removed from the wells by inverting the plate and decanting the contents. A washing procedure followed and repeated 3 to 5 times.

200 µL of IL-6 conjugate were then added to each well, covered with a new adhesive strip and left incubating for 2 hours at room temperature. After a washing step to remove any unbound conjugate (as described above) each well was filled with 50 µL of substrate solution and incubated this time for 60 minutes at room temperature. The amplifier solution (50µL) was then added to each well, covered and left for 30 minutes at room temperature. The reaction was stopped by adding 50 µL of stop solution to each well. Optical density of each well was determined within 30 minutes using a microplate reader (Titertek, Multiskan Plus MKIII, Labsystem, Finland) set to 490 nm.

Duplicate readings for each standard and sample were averaged and the zero standard optical density was subtracted for each one using Excel function (Excel XP, Microsoft Co., USA) (Figure 12).

A linear graph was then created in the same spreadsheet in order to plot optical density of the standards versus the concentration of the standards. A linear regression equation was then calculated where the IL-6 concentration (y) equals optical density reading (x) times linear coefficient (m) plus or minus intercept (z) (y = mx± z). If samples were diluted the reading of the concentration was multiplied by the dilution factor. Intra-assay coefficient of variation was calculated from the formula: Standard Deviation (between duplicate concentration results) times 100, divided by the average concentration result. Mean CV was calculated for each plate and inter-assay CV was calculated in a similar manner.

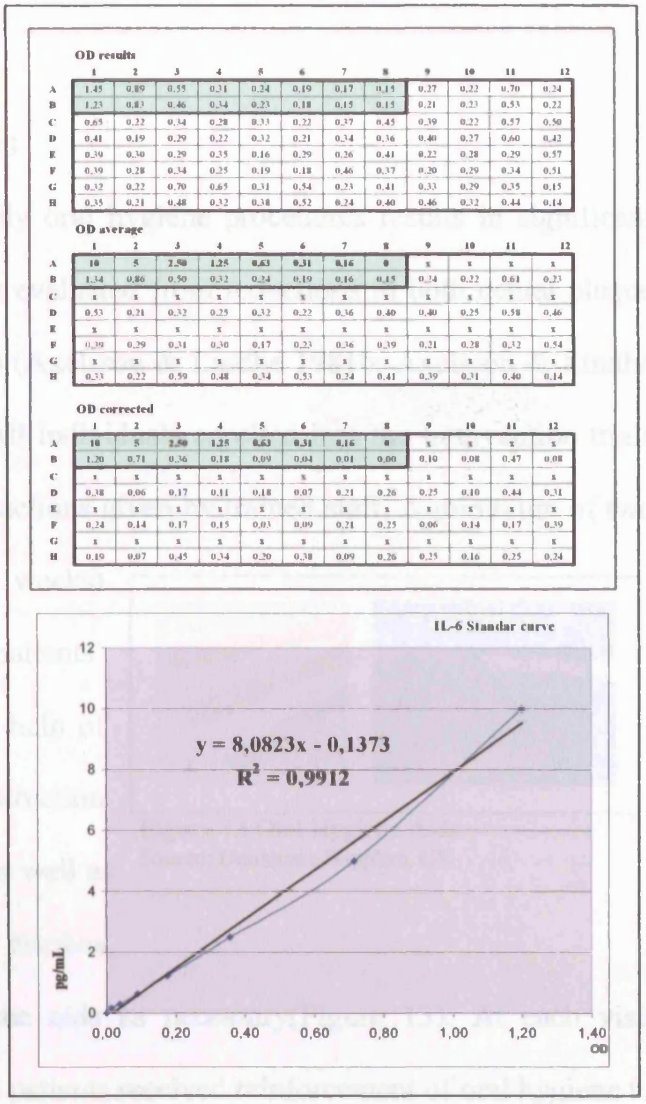


**Figure 12** Optical density readings and IL-6 concentrations calculation.
A First OD readings then averaged and final results after linear regression correction
B Line Plot of OD and IL-6 concentration, regression equation and coefficients used.

## 3.5 PERIODONTAL THERAPY

### 3.5.1 ORAL HYGIENE INSTRUCTIONS

Plaque control by self-performed daily oral hygiene procedures results in significant improvement of oral inflammation as evaluated from reductions in both dental plaque and superficial gingival inflammation (Axelsson & Lindhe 1981b; Axelsson & Lindhe 1981c; Axelsson & Lindhe 1981a). All individuals enrolled into the intervention trials received individual oral hygiene instructions given by trained staff. A minimum of two to three appointments (within two weeks) were always reserved to monitor patients' oral hygiene improvements with the help of plaque disclosing devices, instruction brochures of successful techniques, as well as providing patients with tooth brushes, interdental brushes and other hygiene aids as necessary(Figure 13). At each visit following periodontal instrumentation patients received reinforcement of oral hygiene to predispose for a favourable response (Axelsson & Lindhe 1981b; Axelsson & Lindhe 1981c; Axelsson & Lindhe 1981a).

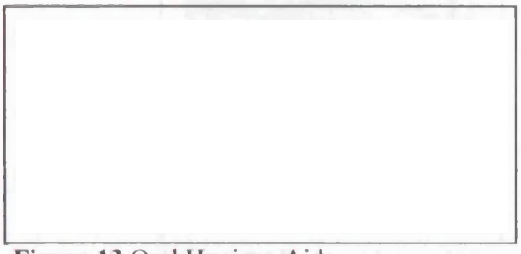

**Figure 13** Oral Hygiene Aids
Source: Dentocare Supplire, UK

### 3.5.2 PERIODONTAL TREATMENT

Based on baseline clinical periodontal parameters and a full set of long-cone periapical x-rays, a proper tooth by tooth periodontal diagnosis was formulated for each individual by a consultant. We investigated for the presence of any carious process with or without pulpal pathology, periapical area of radiolucency, and where a possible resolution was predicted we sent written recommendation to the patients' referring general dental practitioner to provide the necessary dental care. Periodontal instrumentation was performed only when such treatments were completed. We produced a prognostic chart

in which teeth were classified as secure, questionable or irrational to treat according to the extent of periodontal destruction, presence of severe furcation involvement and radiographic residual bone height based on the standard procedures of the Department of Periodontology of the EDI. Briefly, seriously involved teeth with Class III furcation involvement and/or 3+ mobility, and/or with periodontal lesions approaching within 3 mm of the apices were considered hopeless and extracted as part of therapy.

All individuals enrolled received periodontal treatment including complete subgingival debridement with scaling and root-planing under local anaesthesia (2% xylocaine with epinephrine 1:100,000, Astra Pharmaceuticals ltd., Hertfordshire, England).

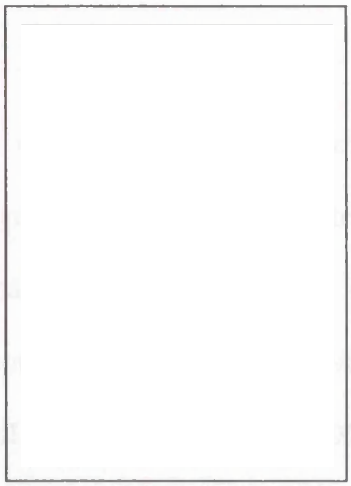

**Figure 14** Piezon Master 400
Source: Ems Nyon, Switzerland

Expert trained periodontists performed the necessary scaling and root planing using exclusively a piezoceramic device (Piezon Master 400, EMS, Nyon, Switzerland) (Figure 14) with continuous water irrigation and a selection of fine subgingival metallic tips (P, PS, PL2, PL3, PL4, EMS, Nyon, Switzerland) (Figure 15). There was no time limitation for treatment: instrumentation had to be completed to the satisfaction of the therapist.

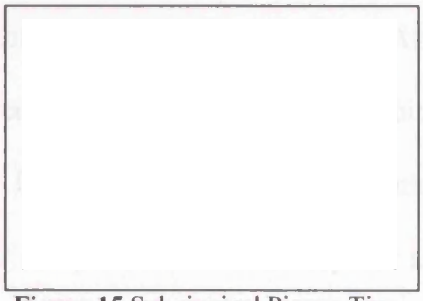

**Figure 15** Subgingival Piezon Tips
Source: Ems Nyon, Switzerland

Particular emphasis on debridement was on furcation sites to meticulously remove any residual calculus since this has proven to result in good clinical results (Matia et al. 1986) (Figure 16). At the treatment visits we also preformed extractions of teeth deemed to be "hopeless" or "irrational to treat" according to standard clinical parameters (eg., with advanced class III furcation involvement and/or mobility of grade III or less than 3mm of remaining periodontal support). In the context of these studies,

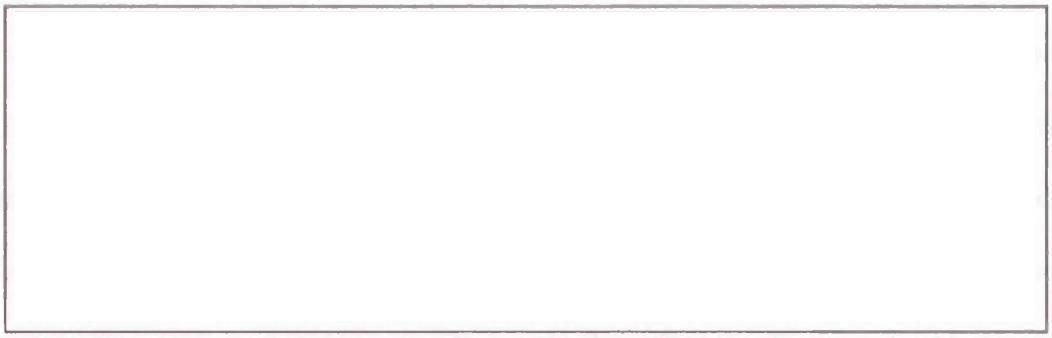

**Figure 16** Subgingival instrumentation by Piezon Master
Source: Ems Nyon, Switzerland

we intended to control the local periodontal infection without using conventional periodontal flap surgery since a series of clinical investigations failed to demonstrate significant differences between thorough subgingival scaling and root planing and periodontal surgical procedures in patients with periodontitis (Becker et al. 1988; Kaldahl et al. 1996b; Kaldahl et al. 1996a; Knowles et al. 1979; Lindhe et al. 1982; Lindhe & Nyman 1985; Pihlstrom et al. 1983; Pihlstrom et al. 1984; Ramfjord et al. 1982; Ramfjord et al. 1987; Westfelt et al. 1985).

At each follow-up visit following periodontal instrumentation, all individuals received standard supragingival prophylaxis together with reinforcement of oral hygiene. After follow-up clinical periodontal parameters were recorded, if any tooth exhibited progression of clinical attachment loss of > 2 mm from baseline, or if any clinical complications occurred (e.g. periodontal abscess) for the patients' own safety the site was treated with an appropriate "rescue" procedure as decided upon by the staff. Any analysis of attachment loss between follow-up visits, included all teeth that had exhibited progressive CAL loss $\geq$ 3 mm.

### 3.6 General Statistical Analyses

All data recorded were entered in computer database on a daily basis by staff blind to any clinical procedures and stored until the end of each trial. Two main statistical software packages were utilized to explore and analyse data (**SAS** version 8.1, Chicago, USA and **SPSS**, v11, Chicago, Illinois USA).

All continuous variables entered were tested for normality by Shapiro-Wilk test. If normality assumptions were met data were reported by means and standard deviations. Parametric statistical tests were utilized to compare different time points outcomes by paired t-test, one-way ANOVA or ANCOVA or repeated ANOVA as appropriate. Whenever the primary outcome was to assess the efficacy of a specific treatment we also performed independent t-test to assess the differences among subgroups (treatment – control). Respective analyses for non normally distributed variables were, Wilcoxon rank-sum paired test, non parametric ANOVA (Kruskal-Wallis), Mann-Whitney test. These variables were reported as median and interquartile ranges.

Association between variables was assessed by non parametric correlation analysis (Spearman). Linear multiple regression analyses were performed to assess any significant contribution on primary outcome measures by all recorded covariates (e.g. age, smoking, gender, ethnicity, body mass index). Multicollinearity tables were also produced and variables affected were removed from the models (backward elimination). Non continuous variables were analysed by Chi-square test or McNemar test, the latter for paired comparisons.

Logistic regression models (binary or multinomial) were also utilized and OR with 95%CI reported.

All analyses were performed with an intention to treat approach. Whenever a patient missed a follow-up or exited early from the trial the last recorded parameters were carried over.

A significant difference was set at $p<0.05$.

# CHAPTER 4.

# SEVERE PERIODONTITIS AND SYSTEMIC INFLAMMATION:

## 4.1 INTRODUCTION

In the last half century, dentists and physicians have emphasized that periodontal infections are localized to the marginal periodontium and that, as such, they rarely have systemic implications in healthy individuals. More recent evidence, however, has indicated that patients with severe periodontitis have increased serum levels of CRP, hyper-fibrinogenaemia, moderate leukocytosis, as well as increased serum levels of IL-1 and IL-6 when compared with unaffected control populations (Amar et al. 2003; Ebersole et al. 1997; Fredriksson et al. 2002; Glurich et al. 2002; Hutter et al. 2001; Loos et al. 2000; Noack et al. 2001; Slade et al. 2000; Slade et al. 2003; Wu et al. 2000b). These data have received considerable attention but fall short of indicating that periodontitis was the cause for the observed serum acute phase responses and thus of establishing that periodontitis may play a role in the etiologic pathway of systemic inflammatory diseases such as the metabolic syndrome and/or atherosclerosis.

Atherosclerosis, with its consequent cardiovascular diseases, represents one of the leading causes of death in the industrialized world. Its aetiological pathway is one of a chronic inflammatory disease. However the stimuli of the invoked inflammatory reaction remain incompletely understood (Ross 1999). In the past fifteen years, many investigators, unable to attribute a large part of cardiovascular events to the well recognized risk factors (cholesterol, lipids, smoking etc), gave support to the old theory that chronic infections may cause atherosclerosis (Leinonen & Saikku 2002).

Several reports and a recent meta-analysis have shown increased odds ratios for cardiovascular events in large populations of subjects harbouring hypothetical triggering chronic infective agents (*H. pylori, C. pneumoniae*). The role of chronic infections on atherosclerotic cardiovascular diseases is now supported by a bulk of validating evidence that has also generated a series of antimicrobial intervention trials to establish

causality in the association (Anderson et al. 1999; Danesh 1999; Fong 2000; Gupta & Camm 1998; Stone et al. 2002).

While the debate on the strength and nature of the association between periodontitis and cardiovascular diseases will continue for some time, recent evidence has changed the definition of periodontitis. Recognized risk factors include the important effect of cigarette smoking, and systemic diseases such as diabetes present also in the panel of the etiological agents for cardiovascular diseases (Genco et al. 2002; Genco 1996; Page & Kornman 1997; Page et al. 1997; Page 1998). Subjects affected by periodontitis share common polymorphisms in specific genes considered important in the regulation of the inflammatory response (Kornman et al. 1999; Kornman 1999; Kornman & Duff 2001). Genetic variations within IL-1, TNF-A, IL-6 genes, in different populations and settings, have been associated also with raised serum concentrations of CRP or other inflammatory markers (Berger et al. 2002; Vickers et al. 2002).

Two mechanistic hypotheses have been proposed regarding the aetiological pathways of the association between periodontitis, systemic inflammation and systemic diseases: one focuses on the chronic infectious burden that periodontitis may represent for the organism due to repeated challenges of either micro-organisms or endotoxin during mastication, tooth brushing etc.; the other sees the diseased periodontium as a source of systemic inflammatory mediators which are dumped into the systemic circulation and are capable of inducing a mild chronic low-grade inflammatory status (Graves 1999; Offenbacher et al. 1981). Either way the possibility that periodontitis can affect systemic health remains a matter of great interest.

We designed a pilot cohort interventional trial whereby severe periodontitis would be treated by machine driven devices (piezo-ceramic) and our aim was to explore the relationship between periodontal infections and systemic inflammation assessed by a cluster of inflammatory markers. We were also interested in investigating whether there

was any dose-effect relationship between these two conditions and whether periodontal therapy would then affect the individual inflammatory basal state. We investigated the possible influence of recognized critical factors for systemic inflammation (age, gender, body mass index and smoking) and genetic variations (polymorphisms in inflammatory and pattern recognition encoding genes) on this association. Finally the relative importance of patient, tooth and site associated covariates on the variability of the clinical periodontal response of this cohort was also evaluated.

## 4.2 MATERIALS AND METHODS

### 4.2.1 EXPERIMENTAL POPULATION

The study was a prospective, single blind pilot intervention trial with 6 months follow-up. Participants were recruited from subjects referred to the Department of Periodontology of the EDI, UCL. Subjects presenting with severe (probing pocket depths greater than 6 mm and marginal alveolar bone loss greater than 30%), generalized (at least 50% of teeth affected) periodontitis were invited to participate in the study without any other systemic infection or disease. These levels of extent and severity of disease were chosen to increase probability of detection of a systemic burden from the local periodontal infection (Slade et al. 2000; Slade et al. 2003). Because of the advanced clinical condition, however, having an untreated control group was not possible (unethical) and a longitudinal cohort design was used to test the principle. Inclusion and exclusion criteria for screening of suitable candidates for the trial have already been reported in section 3.1.2. All patients gave written informed consent; the study had been reviewed and approved by the Eastman/University College London Hospitals joint ethics committee.

### 4.2.2 STUDY OUTLINE

A baseline visit was conducted by a blind calibrated examiner who collected a complete medical history, standard clinical periodontal parameters, blood and microbial samples. A periodontal treatment phase followed and it was carried out by a periodontist. The patients were re-examined 2 and 6

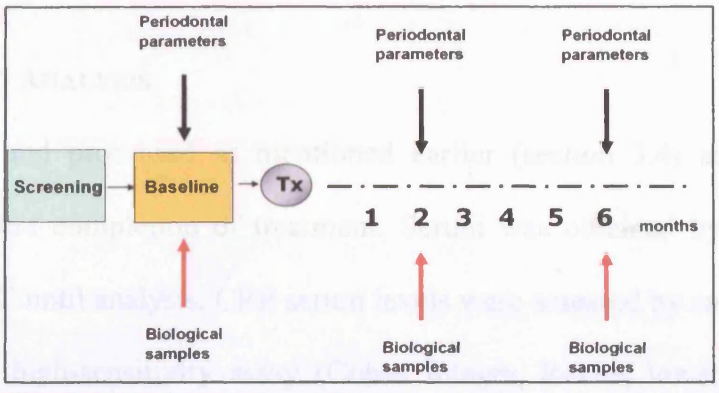


**Figure 17** Study Experimental Design

months after the completion of treatment when the same clinical and serological parameters were collected. After 6 months of therapy a subgroup of individuals, who showed no needs for immediate further periodontal therapy, were monitored for a further six months (total of 12 months follow-up since their initial periodontal therapy). These individuals were re-examined and a blood sample was also collected.

### 4.2.3 Periodontal Parameters and Examiner Calibration

At the three study visits (baseline, 2 and 6 months) site (PPD, REC, CAL), tooth (furcation involvement, mobility) and patient (full mouth BoP and plaque scores) were recorded as described in section 3.2 by one calibrated examiner.

### 4.2.4 Periodontal treatment

Patients were treated for periodontitis by means of non surgical periodontal therapy delivered by a periodontist. Oral hygiene instructions and subgingival scaling and root planing under local anaesthesia were performed using a piezoelectric instrument equipped with appropriate subgingival tips (EMS, Switzerland). The therapist, without limitations in terms of time or number of visits, completed this phase within 1-3 months of the baseline visit. All other dental treatments (extractions of hopeless teeth, restorative and endodontic treatments) were carried out before completion of the periodontal treatment phase.

### 4.2.5 Blood Collection and Analysis

Serum samples were collected and processed as mentioned earlier (section 3.4) at baseline and 2 and 6 months after completion of treatment. Serum was obtained by centrifugation and stored at –70°C until analysis. CRP serum levels were assessed by an automated immunoturbidimetric high-sensitivity assay (Cobas Integra, Roche, lower detection limit of 0.25 mg/L); IL-6 was measured by means of a commercial high-sensitivity ELISA kit (Quantikine HS, R&D System, Minneapolis, lower detection limit of 0.04 ng/L) ( section 3.4.1 for details).

### 4.2.6 GENETIC POLYMORPHISMS ANALYSES

Patients' DNA was extracted from peripheral leukocytes collected at baseline and stored in an EDTA vacutainer tube in a –70°C freezer. A commercial kit was used according to the manufacturer's instructions (Nucleon® BACC2 kit, Nucleon Bioscience, Coatbridge, UK). Blood samples were thawed at room temperature and 30 ml of reagent A (red cell lysis buffer; 10 mM Tris-HCL pH 8; 0.32 M sucrose; 5 mM MgCl2; 1% Triton X-100) added to 10 ml of blood in a 50 ml polypropylene centrifuge tube. The contents were mixed by inverting several times and then centrifuged at 1300g for 5 min to sediment the leukocytes. The supernatant was discarded and 2.0 ml of reagent B (white cell lysis buffer) and 300 µg of proteinase K (Invitrogen, UK) were added to the cell pellet. After vortexing, the tubes were incubated at 37° C for 30 min and 0.5 ml sodium perchlorate (0.5 M) was added, the contents mixed by inversion, then 2.0 ml chloroform was added and mixed again. 300 µl of Nucleon® resin was added and the tubes centrifuged at 1300g for 3 min to separate the phases, followed by removal of the upper aqueous phase containing the DNA, which was transferred to a fresh tube. Two volumes of cold ethanol were added to precipitate the DNA and the DNA was resuspended in 250 µl of sterile water. The DNA concentration was estimated by measuring absorbance at a wavelength of 260 nm using a spectrophotometer.(Pharmacia Biotech, UK) 10 ng of DNA were subsequently used for polymerase chain reaction (PCR) for common polymorphisms in: the IL-1A (Kornman et al. 1997): IL-1B (Kornman et al. 1997; Kornman et al. 1999): IL-6 (Fishman et al.

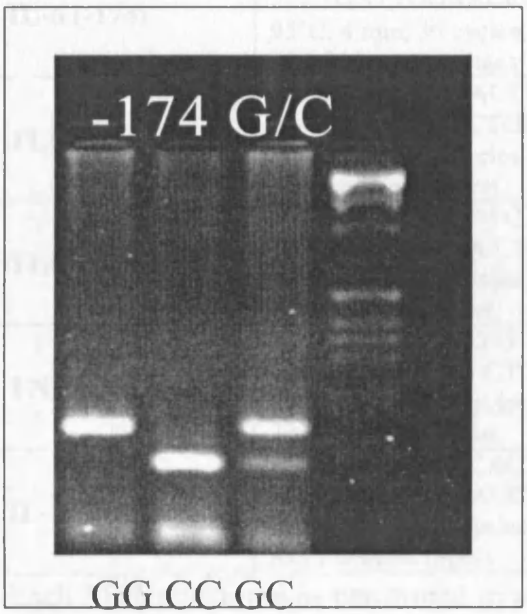


**Figure 18** IL-6(-174) G stands for common allele, C is the rare allele.

1998):TNF-A (Gonzalez et al. 2003). TLR-4 (Lorenz et al. 2001) and IL-10 (Aithal et al. 2001).

**Table 5** Primer sequences, PCR conditions and restriction enzymes used to amplify each marker and determine the genotypes

| MARKER | PRIMER SEQUENCES |
|---|---|
| **IL-1 A (-889)** | 5'-AAGCTTGTTCTACCACCTGAACTAGGC – 3'<br>5'-TTACATATGAGCCTTCCATG – 3'<br>95°C 4 min, 45 cycles; 94°C 1 min, 50°C 1 min, 72°C 1 min,<br>*Nco I* enzyme digest. |
| **IL1 B (-511)** | 5'-TGGCATTGATCTGGTTCATC – 3'<br>5'-GTTTAGGAATCTTCCCACTT – 3'<br>95°C 4 min, 35 cycles; 95°C 1 min, 53°C 1 min, 74°C 1 min<br>*Bco I* enzyme digest |
| **IL-1 B (+3954)** | 5'-CTCAGGTGTCCTCGAAGAAATCAAA 3'<br>5'-GCTTTTTTGCTGTGAGTCCCG 3'<br>95°C, 4 min; 35 cycles; 95°C for 1 min, 67,5°C 1 min, 74°C 1 min<br>*Taq I* enzyme digest. |
| **IL-6 (-174)** | 5' TGACTTCAGCTTTACTCTTGT 3'<br>5' CTGATTGGAACCCTTATTAAG 3'<br>95°C, 4 min; 35 cycles; 95 °C, 45 sec; 63 °C, 1 min; 72 °C, 75 sec<br>*Hsp 2I I* enzyme digest. |
| **TLR4 (-299)** | 5'-GATTAGCATACTTAGACTACTACTACCTCCTCCATG-3<br>5'-GATCAACTTCTGAAAAAGCATTCCCAC-3'<br>95°C, 4 min; 30 cycles; 95°C, 30sec; 55°C, 30sec; 72°C, 30sec.<br>*Nco I* enzyme digest. |
| **TLR4 (-399)** | 5'-GGT TGC TGT TCT CAA AGT GAT TTT GGG AGA A-3'<br>5'-ACC TGA AGA CTG GAG GAG TGA GTT AAA TGC T-3'<br>95°C, 4 min; 30 cycles; 95°C, 30sec; 55°C, 30sec; 72°C, 30sec<br>*Hin*fI enzyme digest. |
| **TNFA(-308)** | 5'-AGG CAA TAG GTT TTG AGG GCC AT-3'<br>5'-TCC TCC CTG CTC CGA TTC CG-3'<br>94 °C, 4 min; 35 cycles; 94 °C, 2 min; 60 °C, 1 min; 72 °C, 1 min.<br>*Taq I* enzyme digest. |
| **IL-10(-627)** | 5'-CTTAGGTCACAGTGACGTGG-3'<br>5'-GTGAGCACTACCTGACTAGC-3'<br>94 °C, 4 min; 35 cycles; 94 °C, 1 min; 50 °C,1 min; 72 °C, 1 min<br>*Rsa I* enzyme digest |

Each PCR reaction was performed in a 25 µl volume containing 1 µl of DNA in buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 500 µM of each dNTPs (dATP, dCTP, dTTP, dGTP), 50 pmol of each of the primers and 1U of taq polymerase (Abgene, UK). The $MgCl_2$ concentration varied for different primers. Sequences of the oligonucleotide primers used for PCR amplification, the size of the predicted PCR products and the PCR amplification program used were described previously and are listed in table 5. Alleles were identified following digestion with restriction endonucleases on a 3% agarose gel containing 0.5 mg/ml ethidium bromide in 1 TBE

buffer at 100 V for 2 h. The bands were visualised using a UV transilluminator (Alpha Innotech Ltd., UK)

### 4.2.7 Microbial Samples and Analysis

Samples of subgingival periodontal plaque were collected at baseline and two and six months after completion of periodontal treatment. Details of the procedure can be found in section 3.3.

Bacterial DNA was isolated from the plaque samples by the use of a commercial DNA extraction kit (Puregene, MI, USA). The DNA was then used as a template to carry out amplification of the bacterial 16S rRNA gene to confirm the presence of bacterial DNA in each sample. This 16S rRNA gene product was then used as a template using species specific primers to amplify regions of DNA specific to one of three different periodontal pathogens by the use of multiplex-polymerase chain reaction (Tran & Rudney 1999). The use of these primers produced three different sized DNA products for *Tannerella forsythensis* (*Tf*) [formerly *Bacteroides forsythus*], *Porphyromonas gingivalis* (*Pg*) and *Actinobacillus actinomycetemcomitans* (*Aa*). PCR products were then visualised on an agarose gel. The presence of a DNA band at the expected size allowed confirmation of the presence of the pathogen within the plaque sample. Two rounds of PCR were performed with specific primers in order to produce a detection limit as low as 10 cells/ml (Gafan et al. 2004; Tran & Rudney 1999).

### 4.2.8 Statistical Analysis

#### 4.2.8.1 General Analysis

As this was a pilot intervention trial, the sample size of this study was not based on formal power calculations but on logistic considerations. No other study was found in the literature at that time to perform a sample size analysis. All data were entered in a computer file, proofed for entry errors and analysed with a two statistical packages (SAS version 8.1, Chicago, USA; SPSS, v11, Chicago, Illinois USA). Continuous

variables are presented as mean and standard deviation or as median and interquartile range if data were not normally distributed. A logarithmic transformation was necessary to normalize IL-6 and CRP serum concentrations prior to any parametric analysis. The Shapiro-Wilk test was used to validate the normality assumptions of both Log[IL-6] and Log[CRP] concentrations. Whenever aggregate variables are reported (average of averages) standard errors are utilized to describe specific variability. Differences in periodontal parameters among various subgroups and between visits were analyzed by one way ANOVA or t-test as appropriate. The Chi-square test was utilized to determine significant association among categorical variables. Significant correlations among inflammatory markers were tested by non parametric analysis (Spearman's rank analysis). Clinical and microbiological outcomes were defined in terms of changes in PPD, REC, CAL and presence of specific periodontal pathogens at different time points. Paired differences in categorical variables were determined by McNemar paired test (e.g. presence or absence of periodontal pathogens after two and six months).

The statistical plan included an analysis on clinical periodontal parameters using both a standard and multilevel (site, tooth, patient) approach. Inflammatory markers basal concentrations and changes after periodontal therapy were analysed with standard statistical procedures including the influence of different genetic polymorphism on basal and post-treatment serum parameters concentrations.

#### 4.2.8.2 Inflammatory Markers

Changes in serum concentrations of CRP and IL-6 following periodontal therapy were used as the primary outcome variables. Due to previously reported intra-individual variations in terms of serum CRP concentration, the association of independent variables with serum IL-6 and CRP concentrations was assessed by linear multiple backward elimination regression analysis. The effects of various genotypes on serum Log[IL-6] and Log[CRP] were analysed by means of ANOVA or independent t-test, as

appropriate. Serum IL-6 and CRP concentrations were preliminarily adjusted for age, body mass index, gender, ethnicity and smoking. The individual response to periodontal therapy in terms of changes in inflammatory markers was assessed by constructing a generalized linear model (using the SAS PROC GLM and REPEATED statement) that included the following confounding factors: age, gender, body mass index, cigarette smoking and cytokine polymorphisms. The null hypothesis was of no changes in CRP and IL-6 concentrations following periodontal therapy. Bivariate and multivariate logistic regression analyses were also used to compare the effect of periodontal treatment on changes in CRP concentrations. Odds ratios (95% CI) were calculated after correcting for potential confounders such as age, gender, ethnicity, body mass index and cigarette smoking.

#### 4.2.8.3 MULTILEVEL ANALYSIS

In order to assess the variance attributable to the different levels of the clinical periodontal data, i.e. the patient, the tooth and the site, a multilevel approach was utilized to fully respect the data hierarchy as well as the nested structure of the variability: site within the tooth, teeth within the patient (Gilthorpe et al. 2000a; Gilthorpe et al. 2000b; Gilthorpe et al. 2001). 3 levels of variability were defined (the patient, the tooth and the site) (see table 6) to construct specific equations essentially representing an evolution of multiple linear regression models (Raudenbush & Bryk 2002). All variables entered into the analysis are listed in table 6. Site and tooth parameters described in section 4.2.3 were included in each model with an additional tooth variable (tooth type = molars, premolars and incisors including canines). The following patient information was also entered into the analysis: age (years), gender, smoking status, body mass index (in $Kg/m^2$), ethnicity, diagnosis (chronic periodontitis vs. aggressive periodontitis, as defined by the American Academy of Periodontology guidelines)(2000b; 2000c; 2000a; Califano 2003). The time of subgingival

instrumentation (in minutes) was noted and aggregate variables were calculated for each subject by averaging specific site information: full mouth plaque (FMPS) and bleeding on probing (FMBS) scores were considered as the percentages of total surfaces which revealed the presence of plaque or bleeding within each subject. The average number of periodontal pockets 5 mm or deeper (NPPK) as well as full mouth averages of PPD and CAL were derived. The carriage of one or two copies of the rare alleles in several polymorphisms was also included (see Table 6 for classification details).

Two separate variance components models (or null model) were constructed using changes in PPD, and CAL between baseline and 6 months (ΔPPD, ΔCAL) as the dependent variables first without inserting explanatory variables. The null model was used to estimate the overall variability of changes in the dependent variables tested and to attribute it to the patient, tooth and site levels. A series of explanatory variables were then entered into each model (covariate model). This further step allowed examination of the relationship between each covariate and the dependent variable.

**Table 6** MLM clinical covariates selection according to three different level of variance (patient, tooth and site)

| | VARIABLE AT BASELINE | CHARACTERISTICS |
|---|---|---|
| Patient | Age | Continuous, years |
| | Gender | Female=0, Male=1 |
| | Ethnicity | Caucasian=1, Non Caucasian=0 |
| | Smoking | Current Smoker=1, Non Smokers=0 |
| | Diagnosis | Chronic Periodontitis=0, Aggressive=1 |
| | BMI | Continuous, kg/m2 |
| | FMBS | Continuous (average full mouth) |
| | FMPS | Continuous (average full mouth) |
| | NPPK | Continuous (average full mouth) |
| | Pg | Presence at Baseline of Pg=1, Absence of Pg=0 |
| | AA | Presence at Baseline of Aa=1, Absence of Aa=0 |
| | Tf | Presence at Baseline of Tf=1, Absence of Tf=0 |
| | Treatment Time | Continuous, minutes |
| | N° Teeth Extracted | Continuous, number |
| | IL-6 (-174) | GG allele=0, GC-CC alleles=1 |
| | IL-10(-627) | CC allele=0, CA-AA alleles=1 |
| | TNFA(-308) TLR-4(-299, -399), IL-1A(-889), IL-1B(-511, +3954), | 1.1 allele=0, 1.2-2.2 alleles=1 |
| Tooth | Mobility | Categorical , Grade 0,1,2,3 |
| | Furcation | Furcation Present=1, Furcation Absent=0 |
| | Type: Incisors, Premolars, Molars | Categorical, Incisors (including canines) =0, Premolars=1, Molars=2 |
| Site | Plaque | Plaque present=1, Plaque Absent=0 |
| | BoP | BoP present=1, BoP Absent=0 |
| | Mesio-Distal | Mesio-distal sites=1, Bucco-Lingual=0 |
| | ΔPPD (Baseline-6 Months) | Continuous, mm |

The normality assumption criterion for inclusion of the dependent variables were verified with the Kolmogorov-Smirnov test (Goldstein 1986; Goldstein 1989; Goldstein et al. 2002). Furthermore an analysis of model residuals was done to confirm the validity of the procedure(Goldstein 1986; Goldstein 1989; Goldstein et al. 2002; Goldstein & Goldstein 1995; Goldstein et al. 2001; Goldstein 2002). A multi-collinearity analysis was performed for each explanatory variable derived. Every linear association was corrected by eliminating the specific variable until the final model fulfilled standard criteria(Bryk & Raudenbush 1992). Regression estimates were

calculated by means of the iterative generalized least square (IGLS) algorithm using dedicated software(*MlwiN v2.0*) (Rasbash et al. 2000). The fit change of each model (–2Log Likelihood) including/excluding explanatory variables was calculated and the significance tested by Chi-square analysis (Goldstein & Goldstein 1995). A significant difference was set to be at $p < 0.05$.

# SEVERE PERIODONTITIS AND MARKERS OF SYSTEMIC INFLAMMATION

### 4.3.1 SUMMARY

Severe periodontitis is associated with elevated inflammatory markers in otherwise healthy populations. However the nature of this association has not been determined. The aim of this specific analysis was to assess whether the degree of response to periodontal therapy was associated with changes in serological markers of systemic inflammation. Periodontal parameters and inflammatory markers CRP IL-6 were evaluated prior, and 2 and 6 months after delivery of standard non surgical periodontal therapy in a cohort of 94 systemically healthy subjects with severe generalized periodontitis participating in a prospective 6 months intervention trial. 6 months after treatment, significant reductions in serum IL-6 ($p<0.001$, median decrease 0.2 ng/L, 95% CI 0.1-0.4 ng/L) and CRP ($p<0.0001$, median decrease 0.5mg/L, 95 % CI 0.4-0.7) were observed. In a multivariate model, serum CRP levels were significantly associated with the outcome of periodontal treatment after correcting for common covariates (age, body mass index, gender, smoking) and polymorphisms in the IL-6 (-174 C/G) and IL-1A (-889) genes. Subjects with above average response to periodontal therapy (<30 residual pockets and <30% of sites bleeding on probing) accounted for the observed improvement in serum CRP after correcting for possible confounders. Control of periodontitis, achieved with non surgical periodontal therapy, significantly decreased serum mediators and markers of acute phase response. Periodontitis may add to the systemic inflammatory burden of affected individuals.

### 4.3.2 INTRODUCTION

Support for the hypothesis that periodontitis driven inflammatory responses are of significance for otherwise healthy individuals is at least threefold: i) periodontitis has been associated with increased odds of cardiovascular events (Scannapieco et al. 2003), of delivering pre-term low birth weight babies (Offenbacher et al. 1998a), and of having sub-optimal control of type II diabetes (Grossi & Genco 1998); ii) the strength of association between periodontitis or other chronic infections and cardiovascular events seems to be of similar magnitude (Danesh 1999); iii) experimental pre-clinical models have indicated that chronic infection with periodontal pathogens leads to thickening of the carotid intima (Lalla et al. 2003; Li et al. 2002) to cause metabolic changes (hyperlipidemia) capable of increasing the risk of atherosclerosis (Jain et al. 2003) and to induce foetal growth restriction (Collins et al. 1994b) .

In recent years an increasing number of epidemiological studies have indicated that subjects with periodontitis may have increased risk of experiencing future cardiovascular events (Beck & Offenbacher 2001). There are however some reports which did not find any significant association (see Table 2). Critics have underlined the fact that both periodontitis and atherosclerosis share commonalities in terms of established risk factors, and that the association, even if established, could be spurious. If the association were firmly established, however, the critical question of its nature in terms of possible causality will remain central to understanding the medical significance of periodontal infections.

Independently of the underlying mechanism(s), systemic inflammation seems to be central for explaining the nature of the link between chronic infections and atherosclerosis (Danesh et al. 2000; Libby et al. 2002; Pearson et al. 2003). In this respect, CRP is generally considered as the most sensitive marker of the APR to

infectious burdens and/or inflammation (Gabay & Kushner 1999). As a consequence of its kinetics, it best describes the inflammatory status of the individual (de Maat & Kluft 2001) (see section 1.1.4). CRP hepatic production is usually elicited by an inflammatory stimulus and mediated through a complex network of cytokines (mainly IL-6) (Ablij & Meinders 2002). CRP serum concentrations in the upper quartiles of normality, moreover, have assumed a significant role as predictors for future coronary events in healthy populations (Ridker et al. 1997). Systemic low grade infections with their moderate acute phase responses may accelerate the formation of atheromatous plaques with consequent increased risk of future cardiovascular events (Danesh et al. 2000). This body of emerging evidence, however, has left unanswered a series of questions on the debated link between chronic infections, e.g. periodontitis, and atherosclerotic events.

The aim of this first analysis was to assess whether the degree of individual response to periodontal treatment was associated with changes in serological markers of systemic inflammation, i.e. CRP and IL-6, in otherwise healthy individuals. Clinical periodontal and microbiological outcomes were also analysed to validate the level of efficacy of periodontal treatment.

### 4.3.3 RESULTS

94 subjects agreed to participate in the study and all reached the 6 months follow up. Overall subjects were 46 ± 9 years of age, 54 % were females, 42 % were current smokers and 26 % had a family history of cardiovascular diseases (see Table 7). The sample population included 75% chronic periodontitis patients while 25% were affected by a generalised aggressive form (2000b; Califano 2003). Their average body mass index was 25.3 ± 3.7 kg/m$^2$.

**Table 7** Baseline Patients Characteristics

| VARIABLE | MEAN ± SD |
|---|---|
| Age (years) | 46 ± 8 |
| Gender | Female 54% |
| Smoking | Current smokers 42% |
| Body Mass Index kg/m$^2$ | 25.3 ± 3.7 |
| Family history of CVD | Positive 26% |
| Periodontal Diagnosis | Chronic 75%<br>Aggressive 25% |
| Nr of teeth at Baseline | 27 ± 3 |
| Nr of teeth extracted | 2 ± 2 |

During the 6 months study period none of the subjects reported any change in diet, medication or smoking habits. After the 6 months follow-up examination 34 individuals were allocated to receive further periodontal treatment whereas 60 individuals were monitored for another 6 months.

The recorded clinical periodontal parameters emphasize the severity and extent of the periodontal infection (Table 8). Participants presented with high levels of gingival inflammation (full mouth bleeding scores of 63.5 ± 16.4%) and severe widespread periodontitis (average of 77 ± 23 periodontal lesions per subject with an average clinical attachment level loss of 3.1 ± 2.5 mm)(Figure 19).

Multiplex PCR detectable levels of specific anaerobic pathogens were found in the majority of the cases as follows: 76.3 % of the subjects were positive for *Tf* 72.8 % for *Pg* and 43.8 % for *A.a*, indicating the high prevalence of infection with recognised periodontal pathogens in this population.

#### 4.3.3.2 Effects of Treatment on Periodontal Outcomes

The clinical periodontal outcomes of treatment are displayed in Table 8. Oral hygiene was significantly improved with 2 and 6 months FMPS averaging about 20% at both 2 and 6 months. FMBS also reached averages of 16 and 17% at 2 and 6 months, respectively. Subjects showed a significant reduction in the number of periodontal pockets (probing 5 mm or more) from 77±23 at baseline to 28±16 at 2 months and 23±15 at the 6 months follow up (P<0.0001, t-test). Changes in full mouth patient averages in terms of PPD, REC but not CAL were also significant at the different time points (P<0.0001, t-test) (Table 8).

**Table 8** Clinical periodontal parameters before and after treatment.

| | Baseline | | 2 Months | | 6 Months | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| **FMPS‡** | 58.04 | 20.70 | 20.90† | 14.29 | 20.08† | 10.42 |
| **FMBS‡** | 63.57 | 16.39 | 15.80† | 11.57 | 17.10† | 11.91 |
| **Nr Pockets*** | 77.08 | 23.23 | 27.82† | 16.36 | 22.91† | 15.04 |
| **PPD mm** | 4.36 | 0.59 | 3.25† | 0.47 | 3.19† | 0.47 |
| **REC mm** | 0.56 | 0.88 | 1.56† | 0.94 | 1.72† | 0.94 |
| **CAL mm** | 4.93 | 1.13 | 4.74 | 1.14 | 4.85 | 1.13 |

*Number of pockets with PPD>4mm. PPD, REC and CAL represent full mouth averages of each subject.
‡ FMPS = Full mouth plaque score, FMBS = Full mouth bleeding score
† P<0.001 t-test compared to baseline.

A significant reduction in the prevalence of *Pg* positive subjects, from 72.8% at baseline to 34.2% at 2 months was achieved (p<0.0001 Mc Nemar test). After six months of standard periodontal therapy the number of individuals positive for all three periodontal pathogens significantly decreased. *Pg* positive subjects remained significantly lower than baseline (36.0 % p<0.0001 Mc Nemar test ). *Tf* and *Aa* samples although did not

reach statistical significance after two months, at 6 months were both significantly reduced (52.6 % p=0.030 and 25.4% p=0.025 respectively).

**Figure 19** Patient clinical features before and after periodontal therapy
A Baseline clinical pictures (frontal and lateral views) B Baseline periapical full mouth radiographical assessment C 6 months clinical pictures (frontal and lateral views)

#### 4.3.3.3 Effect of Periodontal Treatment on Serum Inflammatory Parameters

The baseline median level of CRP was 1.9 mg/L (3.6 IQR, Figure 20) whereas the level of IL-6 was 1.8 ng/L (1.5 IQR, Figure 20. No significant differences in concentrations were found among different groups according to age, gender, periodontal diagnosis (chronic vs aggressive periodontitis) and smoking status.

Both inflammatory markers had a positively skewed distribution curve. A significant decrease in serum IL-6 concentration was found at 2 and 6 months after completion of periodontal treatment (p=0.021, p=0.006, respectively Wilcoxon test). CRP serum

concentration decreased significantly only at the 6 months follow up ($p < 0.0001$, Wilcoxon test, Figure 20). The median change in CRP concentration between baseline and six months was of 0.5 mg/L with a distribution free 95 % confidence interval of 0.4 to 0.7 mg/L.

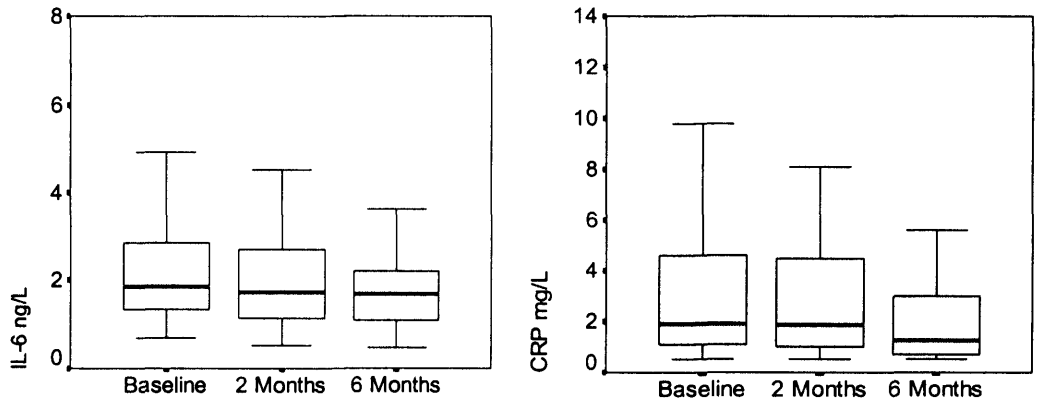


**Figure 20** Box and whiskers plots showing CRP serum levels before and 2 and 6 months following treatment of periodontitis (N=94).
The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. See text for details

The difference in median concentration of IL-6 was 0.18 ng/L (0.02 -0.44 95%CI) between baseline and 2 months and 0.22 ng/L (0.06-0.44 95%CI) between baseline and 6 months (Table 9)

**Table 9** Median differences of systemic inflammatory markers at 2 and 6 months follow up.

| | **Δ Baseline-2 months†** | | | **Δ Baseline-6 Months†** | | |
|---|---|---|---|---|---|---|
| | Median ± IQ | 95% CI* | | Median ± IQ | 95% CI* | |
| **CRP (mg/L)** | 0.1 ± 1.0 | -0.3 | 0 | 0.5 ± 1.3 | 0.4 | 0.7 |
| **IL-6 (ng/L)** | 0.18 ± 1.0 | 0.02 | 0.4 | 0.22 ± 1.0 | 0.06 | 0.4 |

* 95% Confidence Interval distribution free
† Δ = Difference of serum systemic markers between visits.

Improvements in serum IL-6 and CRP concentrations were observed comparing baseline and 6 months values in 73% and 62% of subjects, respectively.

Due to heterogeneity of response to periodontal treatment (in terms of clinical periodontal parameters) and to the presence of well established covariates such as

smoking, age, gender, and body mass index, data were further explored using a repeated measure analysis of covariance model with Log CRP as the dependent variable (Table 10). For the purposes of this analysis subjects were classified in terms of their level of response to periodontal therapy based on the median number of residual pockets and the median percentage of bleeding sites 6 months after treatment. The group with the better response to periodontal therapy was characterised by the persistence of less than 30 pockets 5 mm or deeper and less than 30% bleeding on probing. Among these subjects with the better periodontal response, 79.2% displayed a decrease in serum CRP.

**Table 10** Association of changes in serum CRP and IL-6 with the degree of clinical response to periodontal therapy after controlling for tested confounders

| | **LOG [CRP] (MG/L) N=94** | | **LOG [IL-6] (NG/L) N=94** | |
|---|---|---|---|---|
| | **β ± SE** | **P** | **β ± SE** | **P** |
| Model [a] | | <0.001 | | 0.001 |
| Intercept | -0.859 ± 0.181 | <0.001 | 0.034 ± 0.146 | 0.817 |
| Periodontal Treatment Effect=0 [b] | -0.022 ± 0.012 | 0.069 | -0.007 ± 0.010 | 0.500 |
| Periodontal Treatment Effect=1 [b] | -0.030 ± 0.009 | <0.001 | -0.014 ± 0.007 | 0.041 |
| AGE (years) | 0.009 ± 0.002 | <0.001 | 0.007 ± 0.002 | <0.001 |
| Body Mass Index (Kg/m2) | 0.024 ± 0.004 | <0.001 | 0.003 ± 0.004 | 0.355 |
| SMOKING [smokers vs. non smokers] | 0.112 ± 0.043 | 0.010 | -0.006 ± 0.035 | 0.861 |
| IL-6 (-174 G/C) [CC vs GC] [c] | 0.189 ± 0.065 | 0.004 | 0.010 ± 0.053 | 0.853 |
| IL-1A (- 889) [2.2 vs 1.1] [c] | -0.109 ± 0.056 | 0.054 | -0.033 ± 0.046 | 0.478 |
| IL-1A (- 889) [2.2 vs 1.2] [c] | 0.095 ± 0.054 | 0.082 | 0.021 ± 0.044 | 0.637 |
| IL-1B (-511) [2.2 vs 1.1] [c] | -0.039 ± 0.065 | 0.551 | -0.165 ± 0.054 | 0.002 |
| IL-1B (-511) [2.2 vs 1.2] [c] | 0.017 ± 0.062 | 0.783 | -0.134 ± 0.051 | 0.009 |
| IL-1B (+3954) and GENDER | ... | NS | ... | NS |

[a] Generalized Linear Model testing the association of changes in Log[CRP or IL-6] between 0 and 6 months with the level of response to periodontal treatment, age, gender, body mass index, smoking status, tested cytokine polymorphisms. Model: Log [CRP] Adj. $R^2 = 0.312$; Log [IL-6] Adj. $R^2 = 0.134$

[b] Periodontal Treatment Effect indicates better than median (1) or worse than median (0) outcomes of periodontal therapy in terms of clinical parameters (number of residual pockets less than 30 and full mouth bleeding less than 30%).

[c] The analysis compared the effect of being homozygous for the rare allele (2.2 or CC for IL-6) with either being heterozygous or homozygous for the common allele (1.2 or 1.1, and GG or GC for IL-6)

A highly significant model was constructed that explained 31% of the observed variability in Log CRP in terms of effectiveness of periodontal treatment, age, body

mass index, smoking status, and carriage of specific cytokine genotypes (Table 10). Data indicated that there was a significant interaction between the treatment outcome and the overall CRP levels (baseline, 2 and 6 months), and that the decrease in CRP was significant in those subjects who had the best outcomes in terms of periodontal parameters. The effect was significant after correcting for other known covariates such as age, gender, body mass index and smoking. Furthermore, the analysis indicated that serum CRP concentrations were significantly associated with carriage of specific polymorphic functional alleles in the promoter region of the IL-6 (-174G/C) and IL-1A (-889) genes. Carrying different alleles at the polymorphic sites of IL-1B gene (-511 and +3954) did not reach significance in the model. These polymorphisms were included in the analysis since they have shown to influence basal level of CRP serum concentrations (Berger et al. 2002; Vickers et al. 2002).

A similar analysis using Log IL-6 as the dependent variable indicated that serum IL-6 changes were also associated with the outcomes of periodontal treatment as well as with covariates such as age of the subjects and a specific polymorphism in the IL-1B gene (-511)(Table 10).

When we examined after 12 months a subgroup of 60 subjects, CRP serum concentrations were still significantly lower than their baseline values. This subgroup had a baseline CRP value of 1.8 mg/L (95%CI 1.1-4.3) and showed a significant reduction after 6 months (median decrease 0.4 mg/L, 95%CI 0.0-1.1) in line with the results of the whole population. After 12 months of completion of therapy, the median CRP value was 1.4 mg/L (95%CI of 1.0-2.7) and it was significantly lower than baseline (P=0.003 Wilcoxon rank-sum test) (Figure 21). The significant median change between baseline and 12 months concentrations was 0.4 mg/L with a 95%CI of 0.3-1.2.

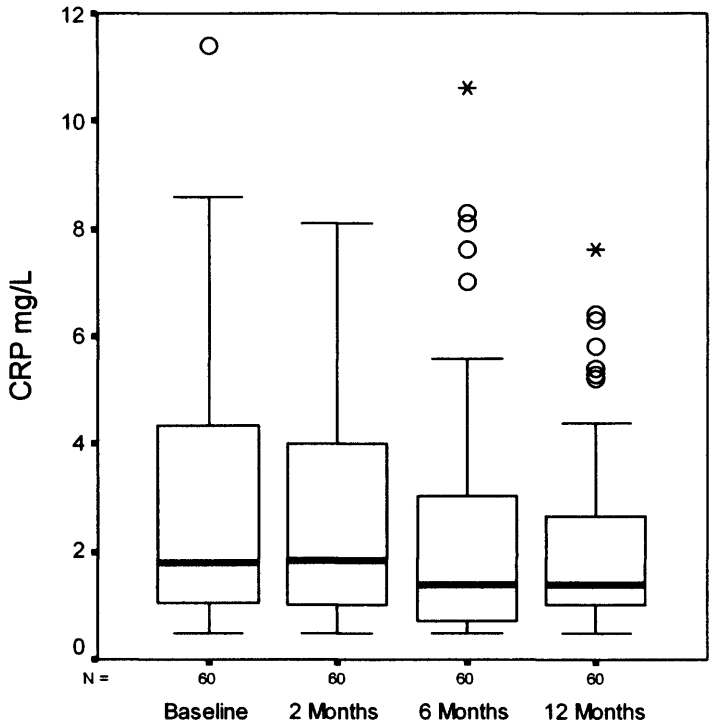


**Figure 21** Box and whiskers plots showing CRP serum levels before and 2 and 6 and 12 months following standard periodontal treatment (N=60)

### 4.3.4 DISCUSSION

Effective control of periodontal infection reduced serum inflammatory markers (CRP, IL-6) in a relatively small population (N=94) with severe generalized periodontitis. Significant decreases in IL-6 were observed already 2 months after therapy, while decreases in CRP were observed only after 6 months. In a subgroup analysis, furthermore, concentrations of CRP remained significantly lower than their baseline levels up to 12 months from the initial periodontal therapy. Of particular relevance, given the preliminary nature of this trial and the lack of a control group, was the observation that reductions in both CRP and IL-6 were significant in subjects who responded better than average to the delivered periodontal therapy. Indeed, 79.2% of the subjects who responded better to periodontal therapy showed an improvement in serum CRP. Within the limits of the cohort uncontrolled design, the data indicated that, in this systemically healthy population, there might be a strong association between periodontitis and the systemic inflammatory status. These data also indicated that maximizing the potential response to periodontal therapy is critical in the context of the design and implementation of a definitive trial.

In this respect it seems relevant to emphasise that serum CRP and IL-6 levels detected at baseline were in the upper quartiles of the current definition of normality when determined with a high sensitivity assay, rather than being in ranges usually associated with acute infections or systemic inflammatory diseases. These data confirm previous observations that otherwise healthy subjects suffering from chronic periodontitis display a moderate increase in systemic inflammation (Loos et al. 2000). This is important in providing a proper interpretation of these findings: in the context of infections physicians have considered frankly elevated CRP values as an indication of a systemic infectious burden requiring appropriate therapeutic measures. More recent data from

longitudinal studies, however, have indicated that acute phase response markers such as CRP and/or IL-6 in the upper distribution of normality seem to be predictive of atherogenesis and cardiovascular events (Danesh et al. 2000; Ridker et al. 1997).

Periodontitis patients have higher CRP and IL-6 levels when compared with matched periodontally healthy populations (see Table 3). However it has become clear that some confounding factors (e.g. cigarette smoking) may be underlying these associations.

We observed a linear positive correlation between the severity of periodontitis and the serum concentrations of CRP, indicating that the effect of periodontitis on systemic inflammation seems to be dose dependent (Table 10).

Renewed interest has been placed in understanding the role of chronic, low grade infections and their associated inflammatory responses. Atherosclerosis is a multifactorial disease. The infection hypothesis has been on the scene since the early years of last century. Many sero-epidemiological studies have supported an association between specific infections and cardiovascular events. *C. pneumoniae, H piilory*, cytomegalovirus, and oral/dental infections have been implicated (Danesh 1999; Leinonen & Saikku 2002). The theory is based upon the presumed noxious effect that these pathogens may have acting directly and/or indirectly (through the inflammatory response) causing: pro-coagulant activity, reduced fibrinolysis, increased leukocyte adhesion, increased cytokine production, and ultimately enhanced LDL and cholesterol deposition in the arterial wall (Libby et al. 2002). Among the possible mechanisms one is that the pathogens may sustain a low grade inflammatory response in the atherosclerotic plaque (mediated by macrophages, endothelial cells, etc.). The intravascular infection may induce an endothelial injury and activate a local inflammatory response. In this vicious circle the activation of inflammatory cells and the production of inflammatory cytokines as well as of adhesion molecules may increase the risk for thrombogenesis. The increased values of systemic inflammation

markers reported in the literature would therefore be an indicator of the atherosclerosis (endothelial damage) progression rather than the effect of distant infections. However a prospective study (Rotterdam Study) indicated that atherosclerosis free individuals with chronic low grade infections had an accelerated progression of atherosclerosis suggesting that there was chronological consistency, with infection preceding the development of atherosclerosis (van, I et al. 2002). Another good example of a clear association between chronic infections (including periodontitis) and atherosclerosis comes from an ongoing prospective study (Kiechl et al. 2001). In atherosclerosis-free individuals the presence of a chronic infection predicted nearly 40 % of new atherosclerotic lesions. This risk was also higher among subjects who showed an increased systemic inflammatory reaction. Whether this was due to particular virulence of pathogens involved or to an abnormal host reaction to them has not been demonstrated yet. More support to this "infectious" hypothesis comes from two of the larger antibiotic secondary prevention trials. A beneficial effect of the antimicrobial therapy in terms of both serum levels of inflammatory markers (reduction at 6 months) and improvement in the endothelial function has been clearly reported (Anderson et al. 1999; Gupta & Camm 1998; Stone et al. 2002; Wiesli et al. 2002) in different population samples (mainly with pre-existing cardiovascular disease). More challenging has been demonstrating that antimicrobials are also effective in reducing the risk of new cardiovascular events. Properly sized randomised controlled clinical trials are currently under way to establish in a definitive way the nature of the association between chronic infections and cardiovascular events. It should be emphasised, however, that these trials will not be able to assess the role of biofilm centred infections such as periodontitis.

Several lines of evidence support the causative role of chronic infections and/or the associated inflammatory responses in atherosclerosis: i) increased odds ratios; ii) chronological consistency between infection and atherosclerosis; iii) data from

experimental models; and in the end iv) emerging results of intervention trials. Data supporting the role of periodontal infections have been limited to the first three lines of evidence. In the majority of studies, subjects with severe generalized periodontitis displayed increased odds ratios of cardiovascular events.

Periodontitis is an infection caused by gram negative bacteria that are organized in a biofilm in a sub-gingival location between the diseased root surface of the tooth and the junctional epithelium. As such, it is relatively insensitive to the effect of systemic antibiotics and its treatment requires, in the first instance, the removal of the biofilm by mechanical professional instrumentation. Following successful treatment, bacterial load is significantly reduced while antibody titres and avidity to the specific pathogens are improved. As a result of these changes, local inflammation significantly decreases and there is a significant improvement in clinical parameters (probing pocket depths, bleeding on probing, etc.).

Reports from large prospective studies have associated periodontitis and tooth loss with an increased risk for carotid atherosclerosis (1mm thickening of the intima) after correcting for possible confounding factors (Beck et al. 2001; Desvarieux et al. 2003). In cell culture studies, *Pg*, one of the most important periodontal pathogens, has shown the ability to invade endothelial cells (Dorn et al. 2000; Genco et al. 1999). Periodontal pathogens have been identified in carotid atheromatous plaques of patients undergoing endarterectomy (Haraszthy et al. 2000). Furthermore *Pg* chronic inoculations in the ApoE +/- mice increased the lipid profiles, enhanced atheroma formation and produced calcifications of the aortal atherosclerotic plaques (Li et al. 2002). The current study expands this evidence to include the results of an initial intervention trial focusing on inflammatory responses. The data generated pose the basis for a controlled intervention trial leaving only the question of whether the delay of the systemic response was to attribute to the nature of the treatment adopted.

Previous to this investigation, only two small trials had reported the impact of non surgical periodontal therapy on systemic inflammation. Christgau et al did not find any effect of periodontal treatment in a mixed small population (20 diabetics and 20 healthy) in terms of changes in CRP levels. One of the reasons may lie in the assay they used for quantification of the acute phase marker (detection limit 2.8 mg/L) and secondly in the extent and severity of periodontitis in their population (Christgau et al. 1998). Non surgical periodontal therapy had a significant effect on white blood counts (specifically in terms of number of neutrophils and thrombocytes) but was strongly confounded by the effect of smoking (Christan et al. 2002).

Since our trial was completed we identified three other studies that investigated the same topic. The first one included a population of 39 patients suffering from periodontitis, all non smokers who were randomly split between a test group (24 subjects) consisting of periodontal therapy and a control group (15 subjects) whose therapy was delayed for three months. No differences in changes were noted comparing CRP concentrations 6 weeks after therapy for the test and 3 months for the control group (Ide et al. 2003). A Finnish group led by Mattila also reported on the effects of periodontal therapy (even including systemic antibiotics in some patients ) in a group of 35 individuals suffering from chronic periodontitis that experienced a reduction in CRP concentrations after 6 weeks of completion of therapy. This difference just approached statistical significance ($p=0.05$) and they also reported high degree of variability in the individual response (Mattila et al. 2002). Both studies did not report any association between clinical response and CRP level changes leaving also some unanswered questions about the efficacy of the treatment adopted. Finally a small case series of 14 individuals showed a significant reduction in CRP and TNF-$\alpha$ serum concentrations after 1 month of periodontal therapy consisting of mechanical instrumentations and repeated adjunctive local antimicrobial (minocycline) therapy (Iwamoto et al. 2003).

The limited number of patients however combined with the presence of important confounding factors (9 of the 14 subjects were type-2 diabetic) do not allow easy interpretation of these results.

One aspect of our results worth emphasising in view of future studies is the indication in the multivariate analysis (Table 10) that CRP decreases following treatment were associated with the clinical outcomes of the delivered periodontal therapy: subjects that had better outcomes than the median at 6 months (in terms of number of residual pockets and bleeding on probing) seemed to account for the observed decrease in CRP after correcting for all the other covariates. Although we lack of any informations of a control untreated group this preliminary observation seems to indicate that there may be a dose dependent effect of periodontal therapy in terms of systemic parameters. Moreover incomplete control of periodontitis (as estimated by persistence of pockets and bleeding on probing) following non-surgical periodontal therapy alone may have precluded achieving an even bigger decrease in serum CRP levels. This observation raises the issue of new end-points in periodontal therapy in the context of prevention of systemic inflammation and possibly atherosclerosis as well as the need to assess the possible benefits of additional periodontal therapy.

The size of the observed improvement in CRP at 6 months is also noteworthy. The reported data indicated that in this population the observed median decrease in serum CRP was 0.5 mg/L. The 95% confidence interval, furthermore, was relatively narrow indicating that the "true" uncorrected median decrease is likely to be in the 0.4 to 0.7 mg/L range. Of further interest was the observation that the magnitude of such difference after periodontal therapy remained constant (0.4 mg/l) in the subgroup of 60 individuals who were re-examined 12 months since their periodontal therapy. Within the limitations of this design, a decrease of this magnitude in terms of CRP is comparable to those observed with some of the most promising medications such as

statins and anti-inflammatory agents (Ridker et al. 1998; Ridker et al. 1999). The medical significance of these changes if proven in larger controlled long-term intervention trials, would substantially decrease the predicted risk for future cardiovascular events based on serum CRP concentrations (Ridker et al. 1998).

Given the low number of reports in which the same microbiological technique has been adopted, we reported a high rate of detection of periodontal pathogens in patients suffering from periodontitis (Gafan et al. 2004; Tran & Rudney 1999). We justify such results based on the fact that the PCR assay used in this study has been shown to detect levels as few as 10 cells of specific periodontal pathogens and therefore much sensitive of any other molecular approaches where the lower detection reported was in the order of $10^2$ to $10^3$ cells (Chuba et al. 1988). Colonization of periodontal pathogens detected with this tecnique in young children has been reported in the range of 30-40 % (Gafan et al. 2004). This would suggest that other factors, such as pathogen numbers might cause disease rather than only presence, giving strength to the ecological plaque hypothesis (Marsh 1994). Furthermore, because patients enrolled in this study suffered from generalized severe forms of periodontitis, this does not allow us to compare our results with previous populations studied due to the difference in the extent and severity of the disease.

Standard periodontal therapy resulted in significant reduction of samples positive for all periodontal pathogens after 6 months. These results are difficult to interpret with other reports due to the different microbiological technique utilized. It has been shown that species are not distributed uniformly throughout the oral cavity, sampling strategy can have affected detection rates and our samples were pooled. Future research is needed in this matter in order to confirm these results.

In this study a significant decrease in serum IL-6 was observed 2 and 6 months after completion of periodontal therapy. A significant decrease in CRP, however, could only

be detected at the 6 months evaluation and 12 months in a small subgroup. This finding is in agreement with the periodontal data reported from literature and described earlier whereby standard periodontal therapy did not induce any change in CRP concentrations after 6 weeks of periodontal therapy (Ide et al. 2003; Mattila et al. 2002).

The results of this study do not offer insights on the possible reasons for this delay. Interestingly, however, looking at the results published from antibiotic intervention trials in patients with cardiovascular diseases this time lag has also been reported (Anderson et al. 1999; Stone et al. 2002). Our original hypothesis, also confirmed by the later studies we performed (see chapter 6 and 7), is that the standard treatment approach utilized, without using any antimicrobial or anti-inflamamtory agent or advanced corrective (periodontal surgery) techniques, is not capable of controlling the local infectious/inflammatory burden and therefore only after a period of time (6 months) a re-established healthy gingival environment produces a significant systemic benefit.

Some limitations in these results have to be reported. Obesity is also a major source of inflammatory mediators' production (de Maat & Kluft 2001; Yudkin et al. 2000; Kluft & de Maat 2001). We cannot exclude that lifestyle or diet changes in our population, even if not reported, could have affected our serological results. Another point could be related to CRP biological variability. Recognized determinants of CRP "physiologic" levels include weight, smoking, statin and hormone replacement therapies, alcohol consumption and antibiotic treatment (Cushman et al. 1999; de Maat & Kluft 2001). There are controversial opinions with regards to biological variability. CRP is thought to have a low biological variation with few outliers (as reported in longitudinal studies) (Macy et al. 1997).

There is no diurnal variation and these outliers may presumably occur for inflammatory stimuli but their clearance should not take more than 3 days (Gabay & Kushner 1999). However a relatively wide variability (~30%) has been also reported (de Maat et al.

1996).(See Section 1.1.4) More research is needed in this matter. In this respect, our data, especially in view of the results observed at 12 months, confirm that CRP is a stable inflammatory marker and open new doors in clinical periodontal research investigating the systemic impact of periodontal therapy. The possibility of having a controlled healthy population sampled at the same time-points of the patients suffering from periodontitis, would better clarify the impact of the CRP individual and seasonal variability on the preliminary outcome we have reported.

The overall limit of this study originated from the cohort design and its preliminary nature. We therefore urge caution in inferring from these results any definite conclusions. These will be addressed by future studies already included in the a priori experimental plan (see Chapter 6 and 7).

# PERIODONTITIS AND CRP ASSOCIATED RISK

### 4.4.1 SUMMARY

Periodontitis has been associated with a moderate systemic inflammatory response. Successful periodontal therapy could decrease serum inflammatory parameters. We aimed to explore the outcomes of periodontal therapy in terms of changes in CRP-associated CVD risk as defined in a recent American Heart Association consensus conference.

At baseline subjects with more severe and widespread periodontitis had a higher chance of having high CRP associated CVD risk (OR 5.6, 95% CI 1.2-27.4). Age and body mass index were also significant in the analysis. After therapy, a significant decrease in the number of subjects associated with a medium and high CRP-associated risk was observed ($p<0.001\chi^2$) with 40 of 94 subjects displaying a decrease in their class of risk. Patients who had a better oral response to periodontal therapy were also more likely to have decreased their inflammatory risk category (OR 4.8, 95%CI 1.4-15.8) after correcting for age, gender, ethnicity and cigarette smoking. This analysis indicated that periodontitis may add to the inflammatory burden of the individual and may result in increased levels of cardiovascular risk based on serum CRP concentrations. These observations will need to be confirmed in a definitive trial. Given the high prevalence of periodontitis in the population, these data would caution physicians to be aware of the possible oral source of an increased inflammatory burden.

### 4.4.2 INTRODUCTION

The systemic implications of chronic and acute infections have attracted increasing attention in the past decade. Common pathogens (e.g. *C. pneumoniae, H. pilori, E. coli*, cytomegalovirus) have been associated with atherosclerosis and cardiovascular events (Epstein et al. 2000; Shah 2002). Reports from preliminary intervention trials assessing the impact of antimicrobial therapy on cardiovascular disease have supported the hypothesis that the observed association may be causal in nature. Some of these pilot studies (ACADEMIC, ROXIS, and STAMINA) showed a significant effect of antibiotic therapy on the occurrence of clinical events (cardiovascular complications) but the matter has not been fully resolved (Anderson et al. 1999; Stone et al. 2002; Wiesli et al. 2002). Furthermore, insufficient evidence has been produced on the potential mechanisms. An intriguing but hypothetical model is based on the systemic inflammatory response subsequent to chronic low-grade infections (Libby et al. 2002). Within this context great interest has arisen from the discovery that even minimally raised concentrations of inflammatory markers may accurately predict future CV events. IL-6 and CRP represent the most sensitive makers used to evaluate the inflammatory status of an individual and appear to be useful predictors for future cardiovascular events in a variety of populations (Pearson et al. 2003; Ridker et al. 1997). Preliminary intervention trials showed a significant effect of systemic antibiotic therapy in reducing systemic inflammatory markers (Stone et al. 2002; Wiesli et al. 2002). A recent joint consensus conference of the American Heart Association (AHA) and the Center for Diseases Control (CDC)has focused on the clinical utility of these markers in the management of CVD risk (Pearson et al. 2003). In addition to the classical risk estimation based on well defined markers (such as smoking, obesity, hyperlipidemia, hypertension, diabetes, age, gender), and due to the available evidence on the role of

inflammation in the pathogenesis of atherosclerosis, the consensus conference identified 3 different risk categories based on serum CRP levels. Pooled epidemiological data from 40,000 subjects have shown that different levels of serum CRP predict future cardiovascular events in otherwise healthy individuals. Based on these observations, subjects with CRP concentrations less than 1 mg/L are considered to be at low risk, while those with concentrations in the 1 to 3 mg/L range are assigned a medium risk level and those with more than 3 mg/L in serum CRP are considered to be at high risk for future cardiovascular disease and events. Periodontitis is a prototype of low grade local infection associated with a moderate systemic inflammatory response. Patients suffering from severe forms of this disease seem to have a perturbation of their systemic homeostasis (Ebersole & Cappelli 2000). Not all periodontitis patients manifest with the same systemic response. It is reasonable to believe that the individual systemic response to periodontitis may be modulated by different genetic (polymorphisms) and environmental (e.g. cigarette smoking or obesity) factors. Periodontitis has been repeatedly associated with increased odds of atherosclerotic events (Danesh 1999). Systemic inflammation may represent the underlying mechanism that links these two common chronic diseases. This analysis focuses on the effects of periodontal therapy on changes in the recently defined CRP-associated CVD risk in a group of otherwise healthy individuals. Results indicated that 6 months following completion of non-surgical periodontal therapy a significant decrease in CVD risk was observed.

### 4.4.3 RESULTS

Categorizing the population according to the AHA/CDC guidelines at baseline, 12 patients were in the low, 47 in the medium and 35 in the high risk group.(Figure 22) A logistic regression model with the AHA/CDC three categories as dependent variable was performed to ascertain the probability of individuals falling in a different category of risk. Estimates and odds ratios (95% CI) were calculated (Table 11). Patients presenting with more widespread periodontitis (expressed by the presence of greater than median number of periodontal pockets 5 mm or deeper) had an OR of 5.6 (95% CI 1.2-27.4, $p<0.04$) and an OR of 3.5 (95% CI 1.2-10.1, $p<0.02$) of being in the highest category of risk compared to the lowest and medium respectively.

**Table 11** Logistic multinomial regression analysis of CRP associated CVD risk categories in patients suffering from severe periodontitis.

| | AVERAGE (CRP<1 MG/L) VS HIGH (CRP>3 MG/L) | | | | MEDIUM (1 MG/L<CRP<3 MG/L) VS HIGH (CRP>3 MG/L) | | | |
|---|---|---|---|---|---|---|---|---|
| | β±SE | P | OR | 95% CI | β±SE | P | OR | 95% CI |
| Constant | 3.911±2.574 | 0.129 | | | 1.879±1.586 | 0.236 | | |
| AGE | -0.153±0.053 | 0.004 | 0.858 | 0.774 to 0.953 | -0.057±0.030 | 0.060 | 0.945 | 0.891 to1.003 |
| GENDER [a] | 0.953±0.811 | 0.240 | 2.593 | 0.529 to12.71 | 0.654±0.520 | 0.208 | 1.923 | 0.695 to5.326 |
| SMOKING [b] | -0.939±0.806 | 0.244 | 0.391 | 0.081 to1.898 | -0.619±0.536 | 0.248 | 0.538 | 0.188 to1.540 |
| ETHNICITY [c] | -0.083±0.840 | 0.921 | 0.921 | 0.178 to4.772 | 0.229±0.548 | 0.676 | 1.257 | 0.430 to3.678 |
| BMI-1 [d] | 2.163±1.072 | 0.044 | 8.696 | 1.064 to71.10 | 1.692±0.670 | 0.012 | 5.431 | 1.460 to20.20 |
| BMI-2 [e] | 1.306±1.016 | 0.199 | 3.692 | 0.504 to27.06 | 0.385±0.583 | 0.509 | 1.470 | 0.469 to4.609 |
| Periodontal pockets [f] | 1.724±.810 | 0.033 | 5.605 | 1.146 to27.41 | 1.262±0.535 | 0.018 | 3.533 | 1.238 to10.08 |

Model P<0.01, -2 Log Likelihood=147.590, Chi-Square 31.325, Pseudo R-Square 0.331
[a] Male vs Female. [b] Smoker vs Non Smoker. [c] Caucasian vs Non Caucasian. [d] Body Mass Index-1: 3rd Tertile (>26.3 Kg/m$^2$) vs 1st (<23.4 Kg/m$^2$). [e] Body Mass Index-2: 3rd Tertile (>26.3 Kg/m$^2$) vs 2nd (>23.4 Kg/m$^2$). [f] Patients who presented with a baseline number of periodontal pockets 5 mm or deeper greater than median (79).

Age and body mass index were the only other significant factors in the model. The analysis accounted for other common confounders such as gender and cigarette smoking.

Non surgical periodontal therapy resulted in a significant improvement of all clinical periodontal parameters after six months. A mean reduction of 57 ± 24 in the number of periodontal pockets greater than 4 mm ($p<0.0001$ t-test) was obtained together with a significant reduction in full mouth bleeding (45 ± 17%, $p<0.0001$ t-test) and plaque scores (38 ± 18%, $p<0.0001$ t-test). Six months after therapy a significant reduction in the number of plaque samples positive for periodontal pathogens ($p<0.001$ Mc-Nemar test) was observed.

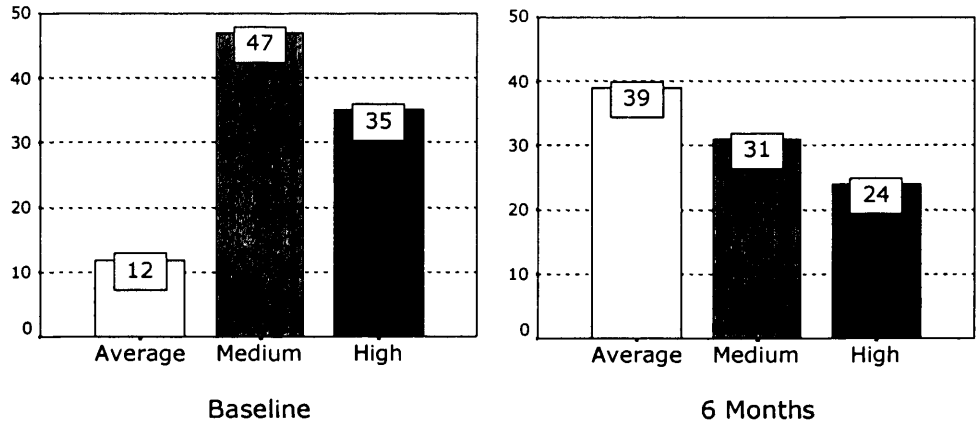


**Figure 22** Bar chart showing baseline and 6 Months frequency (number) of individuals in each CRP-associated CVD risk category.

Serum concentrations of inflammatory markers were also significantly reduced after 6 months for both CRP (median decrease 31%, 54 IQ, $p<0.0001$ Wilcoxon test) and IL-6 (median decrease 12%, 50 IQ, $p<0.0001$ Wilcoxon test).

A significant correlation was observed between the difference in IL-6 and the difference in CRP ($r = 0.38$, $p=0.001$ Spearman rank test).

At 6 months a significant reduction in the number of subjects in the high and medium CRP-associated CVD risk classes was observed ($p<0.0001 \chi^2$ test). 13 subjects moved from the high to the medium category, 25 from the medium to the low, 2 from the high to the low, 4 from the medium to the high and 50 individuals remained in the same category (12 in the low, and 19 each in the medium high categories). Differences in the

number of observations before and 6 months after therapy in each category were significant (low category $p<0.0001$, medium $p<0.05$, high $p<0.05$, Mc Nemar Test).

To further explore the effect of periodontal treatment on CRP-associated CVD risk categories a binary logistic regression analysis was performed with a dependent variable describing the difference in categories defined as follows: reduced CRP-associated CVD risk (1), no change or increased CRP-associated CVD risk (0) (Table12). The analysis controlled for potential confounders (age, gender, ethnicity, smoking, body mass index).

**Table 12** Logistic regression analysis of the CRP-associated risk reduction after periodontal therapy

| | β±SE | P | OR | 95% CI |
|---|---|---|---|---|
| Constant | -0.705 ± 2.355 | 0.764 | 0.494 | |
| AGE | 0.008 ± 0.030 | 0.777 | 1.008 | 0.951 to 1.069 |
| GENDER [a] | -0.931 ± 0.563 | 0.098 | 0.394 | 0.131 to 1.188 |
| ETHNICITY [b] | -0.104 ± 0.256 | 0.686 | 0.813 | 0.298 to 2.220 |
| SMOKING [c] | 1.080 ± 0.603 | 0.073 | 2.944 | 0.903 to9.596 |
| BMI [d] | -0.085 ± 0.076 | 0.260 | 0.918 | 0.791 to 1.065 |
| Extracted teeth (N) [e] | 0.333 ± 0.140 | 0.017 | 1.395 | 1.060 to 1.834 |
| ΔFMPS [f] | 0.029 ± 0.018 | 0.103 | 1.030 | 0.994 to 1.067 |
| ΔFMBS [g] | -0.011 ± 0.018 | 0.540 | 0.989 | 0.954 to 1.025 |
| PPK-EFFECT [h] | 1.557 ± 0.613 | 0.011 | 4.746 | 1.428 to 15.773 |
| Pg change [i] | 0.139 ± 0.536 | 0.796 | 1.149 | 0.402 to 3.286 |
| Tf change [j] | -0.604 ± 0.619 | 0.329 | 0.546 | 0.163 to 1.837 |
| Aa change [k] | 0.363 ± 0.578 | 0.530 | 1.438 | 0.463 to 4.463 |

Model P=0.01, -2 Log Likelihood 94.938, Chi-square 24.819, Pseudo R-square 0.337

[a] Male vs Female [b] Caucasian vs Non Caucasian [c] Smoker vs Non Smoker [d] Body Mass Index ($kg/m^2$) [e] Number of hopeless teeth extracted during treatment [f] Difference in Full Mouth Plaque Scores between 0 and 6 Months [g] Difference in Full Mouth Bleeding Scores between 0 and 6 Months [h] Patients who presented with a reduction in number of periodontal pockets (5 mm or deeper) greater than the median (56 pockets) 6 months after therapy. [i] Patients with undetectable Pg at six months [j] Patients with undetectable Tf at six months [k] Patients with undetectable Aa at six months

Individuals who responded better to periodontal treatment (defined by a pocket reduction at 6 months greater than median) were 4 times more likely (95% CI 1.4-15.8, $p<0.02$) to reduce their risk category compared to those who did not achieve the same clinical response. Participants who also received extractions of hopeless teeth after baseline had an odds of 1.4 (95% CI 1.1-1.8, $p<0.02$) to have a reduction of serum CRP of significant magnitude.

### 4.4.4 DISCUSSION

A significant association exists between the number of periodontal pockets and increased CRP-associated CVD risk according to a recent AHA/CDC classification (Pearson et al. 2003). Otherwise healthy individuals, affected with severe periodontitis had significantly increased odds of being at above average risk (i.e. medium or high risk) for cardiovascular diseases if they presented with more widespread periodontitis defined as higher number of periodontal pockets. This finding is strengthened by the observation that successful non-surgical periodontal therapy resulted in a significant decrease of the CRP-associated CVD risk. Individuals who responded better to periodontal therapy (i.e. those who had above median decreases in the number of periodontal pockets) had increased odds of displaying a decrease in their CRP-associated cardiovascular risk. Accumulating evidence has associated severe periodontitis with increased odds of future cardiovascular events (Danesh 1999). However a clear etiologic pathway has not been proven yet. This analysis suggests a possible role of untreated severe periodontitis on future atherosclerotic processes via systemic inflammation. However, the design of our cohort study do not allow us to conclude that there is a causal relationship between periodontitis and systemic inflammation. Experimental animal data support this hypothesis (Dorn et al. 2000; Jain et al. 2003; Qi et al. 2003; Lalla et al. 2003; Li et al. 2002). A wide range of pathological stimuli (infection, tissue damage) can cause local host production of inflammatory cytokines that may exert a distant effect and alter the normal vascular homeostasis (Libby et al. 2002). This insult, also called endothelial dysfunction, represents a possible mechanism by which chronic infections such as periodontitis, directly via pathogens or indirectly via their products and host inflammatory defences, may initiate and modulate intravascular accumulation of inflammatory cells and lipids (atherosclerosis) (Libby et al. 2002; Shah 2002).

Patients suffering from severe periodontitis have an increased local production of inflammatory cytokines (IL-1β, TNF-α, IL-6) (Gorska et al. 2003; Offenbacher 1996) and a moderate systemic inflammatory response (defined by raised concentrations of CRP, fibrinogen and moderate leukocytosis) (Table 3). Whether this is mainly due to a local excessive production of cytokines that may gain access to the circulation or to a metastatic dissemination of periodontal pathogens and their antigenic products through the bloodstream is still unknown. IL-6 local production in the gingiva may exert its systemic anti-inflammatory activity by stimulating the hepatic synthesis of acute phase proteins in order to protect the host against local pathological stimuli (Irwin & Myrillas 1998). CRP is the prototype of these proteins and it is increasingly assuming the leading role of future predictor for CVD events (Gabay & Kushner 1999; Ridker et al. 1998). Furthermore CRP may play a direct active role in the atherosclerotic process (Pepys & Hirschfield 2003; Ridker et al. 1998). The recent AHA/CDC conference confirmed the potential relevance of small serum changes of the concentrations of this marker in healthy individuals or in those already suffering from CVD. However on the basis of the evidence, the clinical utilization of CRP as a predictive tool for future cardiovascular risk is still considered optional.

This analysis indicated that successful periodontal therapy may reduce CRP-associated cardiovascular risk. Caution is needed in interpreting these results due to the limited number of cases, the variability observed among all participants and the study design. These observations will need to be confirmed in a definitive, prospective, randomized controlled clinical trial. In the meantime given the high prevalence of periodontitis in the population, these data would caution physicians to be aware of the possible oral source of an increased inflammatory burden both in the context of differential diagnosis and in the context of selection of appropriate therapeutic intervention.

# GENETIC DETERMINANTS OF THE LOCAL AND SYSTEMIC INFLAMMATORY RESPONSES TO CHRONIC PERIODONTAL INFECTIONS

### 4.5.1 SUMMARY

The inflammatory response to chronic infections such as periodontitis may be central to the systemic implications of these diseases. This analysis aimed to investigate the degree of exposure between periodontitis and systemic inflammation and explore the possible association between specific gene polymorphisms and the systemic inflammatory response in a cohort of individuals suffering from severe generalized periodontitis. 94 subjects with periodontitis were genotyped for polymorphisms in IL-1A (-889), IL-1B (-511, +3954), TNF-A (-308), IL-6 (-174) and TLR-4 (-299,-399) genes. We found that the genotypes for IL-1A or IL-6 are associated with higher levels of serum IL-6 ($P<0.03$) and serum CRP ($P<0.05$), similarly the TNF-A genotype is associated with higher levels of serum IL-6 ($P<0.05$) after correction for age, body mass index, gender, ethnicity and cigarette smoking. Systemic inflammatory responses are higher in severe periodontitis patients carrying rare alleles for functional inflammatory gene polymorphisms. These results suggest that cytokine genotypes are important determinants of the systemic inflammatory response in subjects with periodontitis. Genetic polymorphism therefore, may in part explain the reported association between periodontitis and systemic disease.

### 4.5.2 INTRODUCTION

Periodontitis is an opportunistic mixed infection of the tooth supporting tissues. Its aetiology recognizes a synergistic role between specific anaerobic bacteria organised in a biofilm, sub-gingival dental plaque, and an exaggerated inflammatory response in susceptible individuals. Periodontitis is highly prevalent affecting 10 to 15% of the adult population and, represents the major cause of tooth loss in adults leading to long term disability and increased treatment needs (Albandar & Rams 2002; Papapanou 1996; Papapanou 1999; Ridker et al. 1998).

As such chronic periodontitis is a recurrent disease that alternates between active and quiescent phases. The individual inflammatory response induced by the pathogens seems to play a critical role in the disease pathogenesis (Genco & Slots 1984). High levels of inflammation of the periodontium have been associated with the actual progression of the disease (Offenbacher et al. 1981). Evidence has indicated that specific hyper-inflammatory traits are present in patients affected with severe periodontitis (Offenbacher 1996). These include carriage of the rare alleles of functional polymorphisms in the interleukin-1 gene complex (Kornman et al. 1997) and TNFA gene (Qian et al. 2002).

In the last decade, the discovery of an association between periodontal infections and other diseases (pre-eclampsia, atherosclerosis, diabetes, and respiratory diseases) has placed increased emphasis on the systemic implications of periodontal infections (Danesh 1999; Shay 2002). Recent evidence has indicated that patients with severe periodontitis have a perturbation of their systemic inflammatory status manifested by increased serum levels of IL-6, IL-1, CRP, fibrinogen and moderate leukocytosis when compared with unaffected control populations (Table 3). Independent of the underlying mechanism(s), the best approach to test this hypothesis would be to explore all possible

influential factors acting on the systemic inflammatory response in patients suffering from periodontitis. In this context, IL-6 and CRP are generally considered to be the most sensitive markers of the acute phase response to infections and inflammation (see section 1.1). The production of IL-6 is elicited at the site of the infection directly by bacterial (LPS) or cytokine (TNF-α, IL-1β) stimulation (Irwin & Myrillas 1998; Shay 2002; Graves 1999). IL-6 as a pleiotropic cytokine is responsible for the synthesis of CRP and other acute phase proteins in order to contain the local infection (see section 1.1.3). Serum concentrations of both inflammatory markers have also assumed a significant role as predictors for future cardiovascular events in healthy populations (see section 1.2.3). Significant evidence indicates that the individual inflammatory response is modulated by specific polymorphisms at inflammatory genes. Genetic variants in the IL-1A (-899) IL-6 (-174) and TNF-A (-308) genes have been linked to enhanced systemic inflammatory responses based on serum levels of specific mediators (Gonzalez et al. 2003; Shay 2002; Fishman et al. 1998; Vickers et al. 2002). Little is known, however, about the relationship, if any, between cytokine polymorphisms and the systemic inflammation associated with periodontal infections.

The aim of this analysis was to investigate first whether there was a linear positive association between severity of periodontitis and magnitude of individual systemic inflammation defined by serum concentrations of IL-6 and CRP. Second we explored the possible association between specific cytokine polymorphisms and the systemic inflammatory response (estimated as serum CRP and IL-6) in individuals suffering from severe generalized periodontitis. The data indicated that, in patients with severe periodontal infections, increased serum IL-6 was associated with carriage of allele 2 for IL-1A (-899), TNF-A (-308) and IL-6 (-174), while serum CRP was associated with allele 2 for IL-1A (-889) and IL-6 (-174) after correcting for age, body mass index, gender, ethnicity and cigarette smoking.

### 4.5.3 RESULTS

#### 4.5.3.1 SYSTEMIC INFLAMMATION AND SEVERITY OF PERIODONTITIS

Median serum IL-6 was 1.8 ng/L (1.5 IQ) while CRP was 1.9 mg/L (3.5 IQ). The serum concentrations of the two inflammatory markers were significantly correlated ($r=0.34$ $p<0.001$, Spearman's rank correlation test). No significant differences in these markers were observed among subgroups categorised by ethnicity, gender, smoking or bacterial detection.

Logarithmic transformations of IL-6 concentrations were significantly associated with age and periodontitis severity assessed as the individual mean PPD (pockets greater than 5 mm) by backward elimination multiple regression analysis (Table 13).

**Table 13** Linear Regression Model predicting IL-6 concentrations

| | LOG[IL-6] [a] | T | P |
|---|---|---|---|
| | UNSTANDARDIZED COEFFICIENTS [b] ± SE | | |
| **Intercept** | -1.231 ± 0.452 | -2.724 | **0.008** |
| **Age** | 0.008 ± 0.003 | 2.618 | **0.011** |
| **PPD 6MM** [c] | 0.117 ± 0.053 | 2.203 | **0.031** |

[a] Adj R square 0.344, F<0.0001. [b] Unstandardized coefficients are expressed as Log of IL-6.
[c] Full mouth average of probing pocket depths greater or equal to 6 mm.

Similarly, Log CRP concentrations were significantly associated with age, body mass index, Log concentration of IL-6 and severity of periodontitis evaluated as the individual average full mouth clinical attachment level (Table 13).

**Table 14** Linear Regression Model predicting CRP concentrations

| | LOG [CRP] [a] | T | P |
|---|---|---|---|
| VARIABLE | UNSTANDARDIZED COEFFICIENTS [b] ± SE | | |
| Intercept | -0.784 ± 0.258 | -3.043 | **0.003** |
| **Log [IL-6] ng/L** | 0.394 ± 0.146 | 2.689 | **0.009** |
| **CAL mm** [c] | 0.082 ± 0.030 | 2.774 | **0.007** |
| **BMI Kg/m$^2$** [d] | 0.023 ± 0.009 | 2.658 | **0.009** |

[a] Adj R square 0.292 F<0.0001. [b] Unstandardized coefficients are expressed as Log of CRP. [c] Full mouth average clinical attachment level (mm). [d] Body Mass Index.

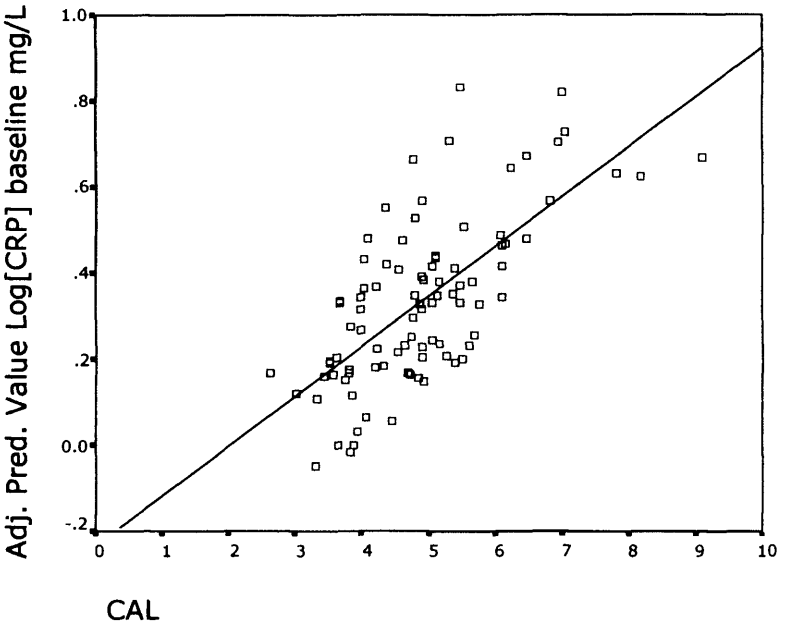


**Figure 23** Scatter plot of CAL levels against adjusted Log CRP concentrations.
Values produced after linear regression model (see Table 13)

### 4.5.3.2 Gene Polymorphisms and Systemic Inflammation in Periodontitis

The observed allele frequencies and genotypes of the tested cytokine polymorphisms are reported in table 15 and are in accordance with previous reports (see section 4.2.6).

**Table 15** Genotype and Allele Frequencies of the Tested Polymorphisms

| Polymorphism | 1.1 N (%) | 1.2 N (%) | 2.2 N (%) | Allele 2 Frequency |
|---|---|---|---|---|
| IL-1A (-889) | 36 (39) | 35 (37) | 22 (24) | 0.45 |
| IL-1B (-511) | 35 (37) | 46 (49) | 13 (14) | 0.36 |
| IL-1B (+3954) | 63 (67) | 25 (27) | 5 (6) | 0.21 |
| TNF-A (-308) | 67 (74) | 22 (25) | 1 (1) | 0.15 |
| IL-6 (-174) | 52 (56) | 29 (31) | 12 (13) | 0.27 |
| IL-10 (-627) | 49 (55) | 34 (38) | 6 (7) | 0.26 |
| TLR-4 (-299) | 76 (87) | 11 (13) | - | 0.07 |
| TLR-4 (-399) | 86 (94) | 6 (6) | - | 0.03 |

IL-6 serum concentrations (adjusted for age, body mass index, gender, ethnicity and smoking) were compared in subjects homozygous for the common alleles and subjects carrying one or more copies of the rare allele (Figure 24). Periodontitis patients carrying one or two copies of the rare allele of IL-1A (-889), TNFA (-308) or IL-6 (-174)

polymorphisms displayed significantly higher serum IL-6 concentrations. The observed mean difference in serum IL-6 between 1.1 subjects and 1.2 or 2.2 subjects was 0.9 ± 1.1(SE) ng/L for IL-1A (P=0.031), 0.8 ± 1.1(SE) ng/L for TNF-A (P<0.001) and 0.8 ± 1.1(SE) ng/L for IL-6 (P<0.001). No significant differences in serum IL-6 were observed in subjects with different genotypes in the promoter region of IL-1B (-511, +3954) and TLR-4 (-299, -399 – data not displayed in the figure) in this population.

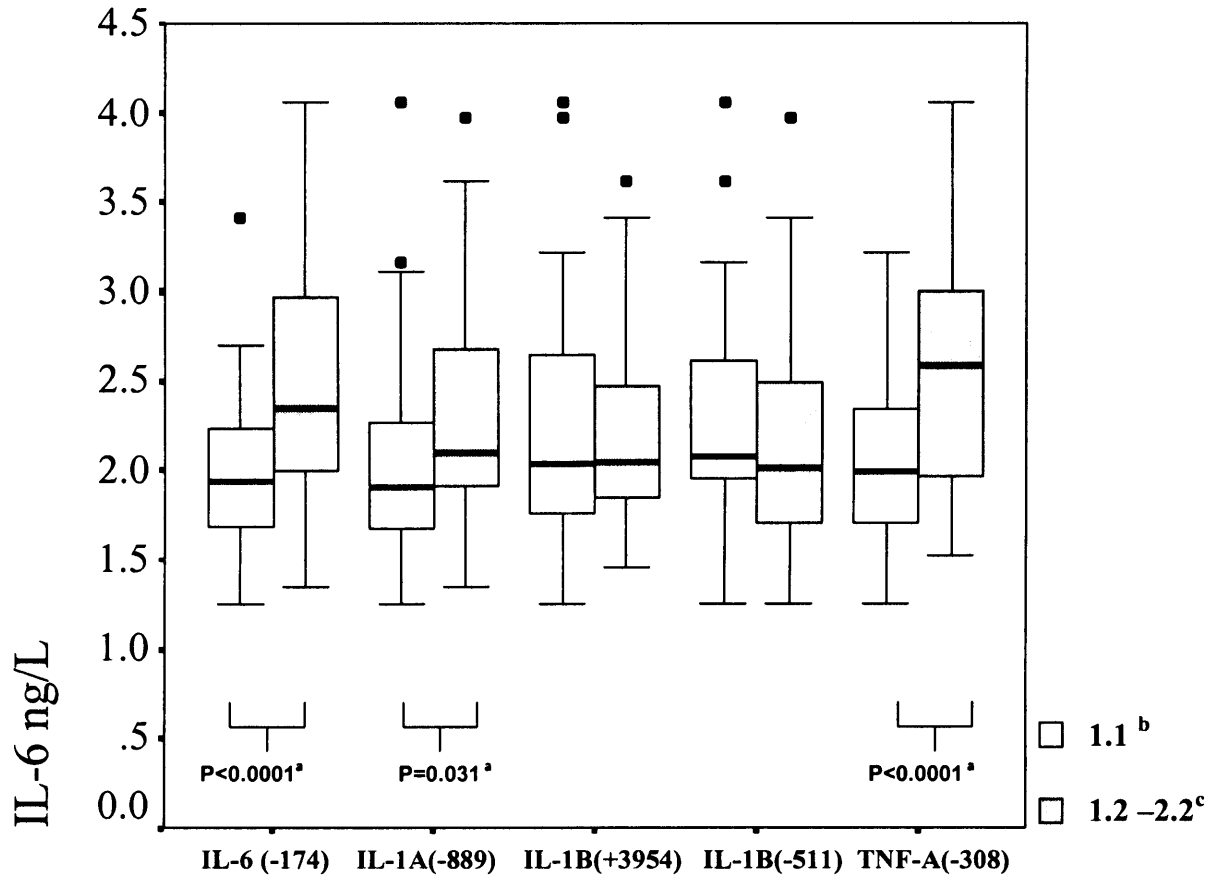


**Figure 24** Box and whiskers plots show different concentrations of IL-6 according to different genotypes. Box and whiskers plots show different concentrations of IL-6 according to different genotypes in inflammatory gene polymorphisms after controlling for age, gender, ethnicity, body mass index and smoking. The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Black dots refer to outliers. For the exact number of subjects in each subgroup refer to Table 15.
[a] Significantly different comparing 1.1 to 1.2 and 2.2 (t-test). Statistics performed on logarithmic values whereas in the figure back transformed values are reported for clarity.
[b] GG for IL-6 (-174) and TNF-A(-308)
[c] GC-CC for IL-6 (-174), GA/AA for TNF-A(-308)

A similar analysis conducted to compare serum CRP concentrations (adjusted as for IL-6) indicated that subjects carrying one or two copies of the allele 2 at IL-6 (-174) and IL-1A (-889) had significantly higher CRP serum levels than subjects homozygous for

1.1 (Figure 25). The observed difference in serum CRP between 1.1 subjects and 1.2 or 2.2 subjects was 0.8 ± 1.1(SE) mg/L for IL-1A (P=0.048) and 0.8 ± 1.1(SE) mg/L for IL-6 (P=0.023). All other polymorphisms tested were not significant.

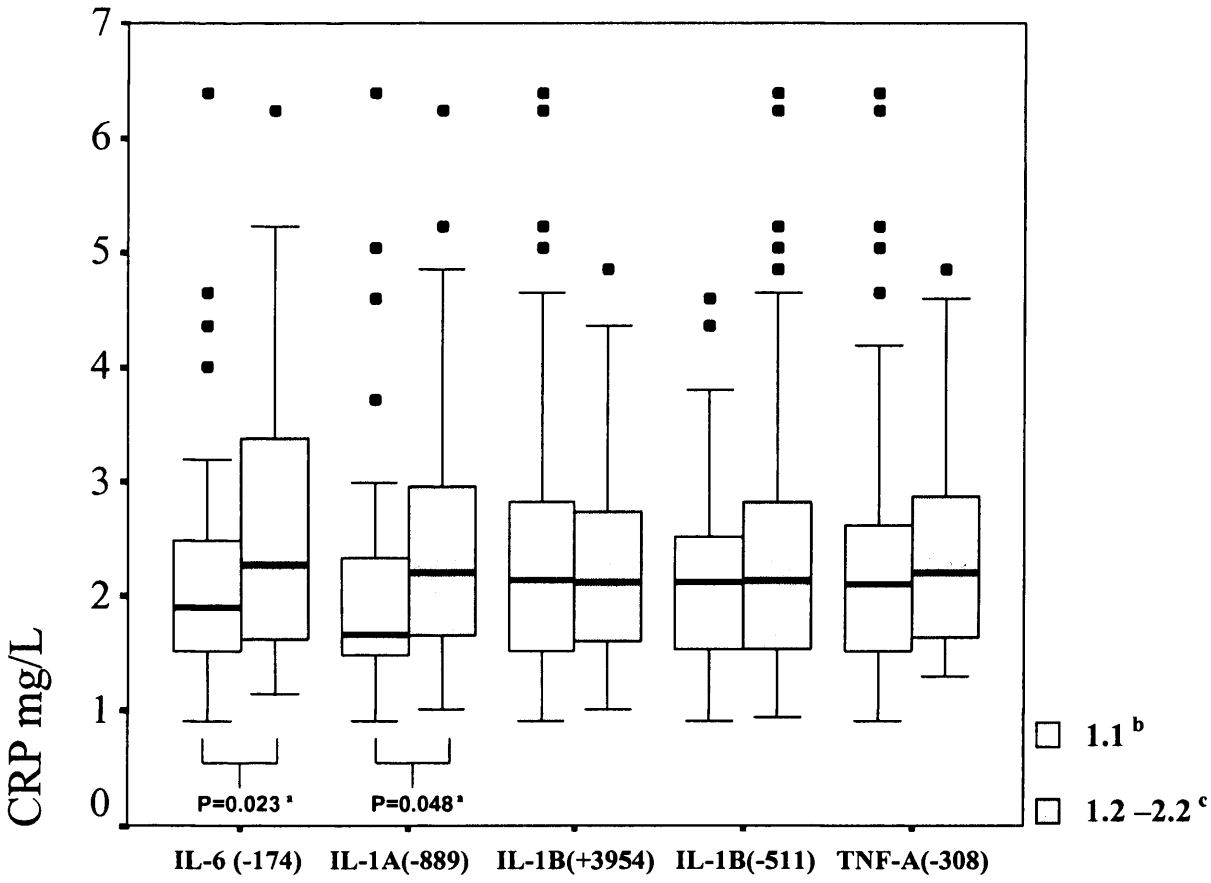


**Figure 25** Box and whiskers plots show different concentrations of CRP according to different genotypes Box and whiskers plots show different concentrations of CRP according to different genotypes in inflammatory gene polymorphisms after controlling for age, gender, ethnicity, body mass index and smoking. The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Black dots refer to outliers. For the exact number of subjects in each subgroup refer to Table 15.
[a] Significantly different comparing 1.1 to 1.2 and 2.2 (t-test). Statistics performed on logarithmic values whereas in the figure back transformed values are reported for clarity.
[b] GG for IL-6 (-174) and TNF-A(-308)
[c] GC-CC for IL-6 (-174), GA/AA for TNF-A(-308)

### 4.5.4 DISCUSSION

Patients with severe periodontitis recruited for this trial showed a positive association between serum inflammatory markers and clinical measures of extent and severity of periodontitis independent of age, body mass index, gender and cigarette smoking. Serum IL-6 concentrations were also significantly associated with the carriage of allele 2 for functional polymorphisms in the IL-1A, TNF-A and IL-6 genes after correcting for age, gender, ethnicity, and smoking status. Similarly, serum CRP concentrations were associated with allele 2 in the IL-1A and IL-6 genes. In this subject group no medical condition other than severe generalised periodontitis was detected, furthermore no subjects reported taking any systemic medication.

Previous investigations have associated the severity of periodontitis with the carriage of a composite genotype that included allele 2 of the IL-1A (-889) and IL-1B (+3954) (Kornman et al. 1997). This composite genotype has also been associated with the detection of higher concentrations of IL-1 in both the gingival tissues and the inflammatory exudates originating from the marginal periodontium (Engebretson et al. 1999). The current data extend these observations indicating that specific cytokine polymorphisms are associated with the extent of the systemic inflammatory response to periodontitis.

These genetic variations might produce an imbalance between agonist inflammatory mediators and influence the individual response to pathogens as well as to the treatment. Berger et al in a population with significant atherosclerosis found that the 3954 hyper-inflammatory type 2 allele for IL-1B markedly influenced (2-3 fold increase) baseline CRP serum levels. A similar but weaker effect was found in subjects carrying allele 2 at the polymorphic site (4845) of the IL-1A gene that is in 99% agreement with the IL-1A (-889) polymorphism (Berger et al. 2002). In the present report, this specific genotype

(IL-1A -889) showed a significant association with serum CRP levels whereas differences in the 3954 loci did not reach statistical significance. Furthermore this study detected a significant association between IL-6 (-174 G/C) polymorphisms and both pre- and post-treatment serum values of CRP. CRP and IL-6 baseline levels have been associated already with this specific genotype. A recessive effect of the C allele on the mean levels of IL-6 was reported (Margaglione et al. 2001) whereas a dominant effect of the same was associated with increased baseline CRP levels (Vickers et al. 2002). We also found a significant association between the -511 IL-1B polymorphism and serum IL-6 levels.

An expanding literature has associated the carriage of specific cytokine polymorphisms with the outcome of a variety of infections (Emonts et al. 2003; Holmes et al. 2003; Tabrizi et al. 2001). This is proposing a novel explanation of the individual variability in response to similar pathological stimuli. Genetic polymorphisms of antigen recognition pathways (TLR) and pro-inflammatory cytokines (IL-1, TNF-α, IL-6) have been extensively analysed (Waterer & Wunderink 2003). These polymorphisms often have been associated with increased local and systemic cytokines production. CRP synthesis rate, that represents a reliable marker of the persistence of a noxious stimulus, has also been linked to IL-1 and IL-6 polymorphisms in different populations and settings(Berger et al. 2002; Pankow et al. 2001; Vickers et al. 2002). The mechanisms, however, are still being investigated.

A recent report has stressed the role of cytokine polymorphisms, and the IL-6 (-174) in particular, in determining the levels of systemic inflammation associated with chronic low grade infections leading to an increased risk of atherosclerosis (Georges et al. 2003). This study indicated that the association between atherosclerosis and pathogen burden was modulated by the IL6/G -174C polymorphism and appeared to be mediated by variations in serum IL-6 levels (rare allele showing the highest concentrations). In

the current study, serum IL-6 levels were associated with the tested genotypes, and highly correlated with serum CRP levels. These data and previous findings support the hypothesis that the systemic inflammatory responses to chronic infection might be modulated by host genetic factors and that the local production of inflammatory mediators determines the serum concentration, of acute phase reactants such as CRP. Caution is needed, however, in interpreting these results given the relatively small number of subjects included.

In recent years, chronic, low grade infections such as periodontitis have been associated with systemic inflammatory diseases like atherosclerosis (Danesh 1999). A variety of mechanistic models linking chronic infections with an increased risk of atherosclerosis have been proposed, but inflammation is central to all of them. If inflammation is the key mechanism of this association, the present findings indicate that the risk of developing atherosclerosis may be modulated by an individual's genotype and that carriers of the rare alleles of specific cytokine genes may represent a higher risk group for developing systemic disorders associated with chronic infections like periodontitis.

# RELATIVE CONTRIBUTION OF PATIENT, TOOTH AND SITE-ASSOCIATED VARIABILITY ON THE CLINICAL OUTCOMES OF SUBGINGIVAL DEBRIDEMENT

### 4.6.1 SUMMARY

The objective of this analysis was to assess the relative contribution of patient, tooth and site associated variables on changes in PPD and CAL following delivery of a standard non surgical phase of periodontal therapy.

The relative contribution of patient, tooth and site associated variables was evaluated with a hierarchical multilevel analysis. 80% of variability in PPD and CAL reductions was attributed to site level parameters, while 12% (14% for CAL) was at the tooth and 8% (7% for CAL) at the patient level. The multilevel analysis associated PPD reductions with patient factors [cigarette smoking status and carriage of the rare allele of a specific polymorphism for the IL-6 gene], tooth factors (tooth mobility and tooth type), and site factors (mesial and distal location, as well as baseline probing depth). In particular, smokers and carriers of the C allele (-174, IL-6) had significantly less reductions in probing pocket depths. Further confirmation of their effect was the discovery of a similar effect on CAL changes. Significant determinants on changes of CAL were also ethnicity (caucasian), polymorphisms of TLR-4 and TNF-a genes and the presence of a periodontal pathogen (*Pg*). Incisors and canines responded better than premolars and molars. A dose dependent effect of mobility was observed: teeth with higher baseline mobility resulted in significantly greater decreases in PPD. Presence of furcation involvement at baseline negatively affected changes in CAL. At the site level, greater reductions were observed at interdental sites (compared to facial or oral), and at deeper sites (1.2 mm for 4-5 mm pockets and 2.4 mm for 6 mm or deeper).

These data provided an estimation of the relative contribution of site, tooth and patient associated variables in terms of PPD and CAL reductions following a standard course of machine-driven subgingival debridement.

### 4.6.2 INTRODUCTION

The initial, infection control phase of periodontal therapy is the critical component in the treatment of periodontitis. Summaries of the evidence provided at the World Workshop of 1996 and the European Workshop on 2002 indicate that the combination of sub-gingival debridement with oral hygiene instruction is effective in reducing probing pocket depths and inflammation (Cobb 1996; Van der Weijden & Timmerman 2002). The expected magnitude of the observed decrease in pocket depth at the site level ranged between 1.3 mm in shallow (4-5 mm) and 2.2 mm in deep ($\geq$6 mm) pockets. (Cobb 2002; Cobb 1996)

Traditionally, periodontists have performed their sub-gingival debridement using primarily hand instruments but improvements in machine-driven instruments over the last decade have significantly expanded the scope for using sonic, ultrasonic or piezoelectric instruments with fine tips specifically designed to reach the root surfaces at sites with deep pockets. A recent systematic review discussed at the 2002 European Workshop compared the clinical efficacy of sub-gingival debridement performed with hand or machine-driven instruments (Tunkel et al. 2002). Results indicated that "with respect to clinical outcome measures, the available data do not indicate a difference between machine-driven and manual debridement in the treatment of chronic periodontitis for single-rooted teeth". The retrieved evidence to support this statement, however, was considered to be "not very strong". From the systematic analysis of the data, it also emerged that machine-driven sub-gingival debridement to the satisfaction of the treating clinician required 37% less time than hand instrumentation.

With respect to the external applicability of these observations, some evidence indicates that the level of response may be lower in specific patient groups (e.g. smokers, diabetics), and at areas that are difficult to treat (e.g. molars, furcations, very deep

pockets). The validity of some of these data, however, has been questioned since the majority of the classical studies did not provide patient based data and did not account for the lack of independence of the outcomes of different sites/teeth in the same patient (Tonetti & Cobb 2002).

It is also noteworthy that, to date, no research has addressed the issue of the relative importance of patient, tooth and site specific parameters in determining the observed variability of outcomes of sub-gingival debridement.

The objective of this analytical approach was to assess, using a multi-level analysis, the relative contribution of patient, tooth and site-associated factors in determining the clinical outcomes of sub-gingival debridement performed with a machine-driven instrument.

### 4.6.3 RESULTS

A total of 2589 teeth and 15498 sites were included in the multilevel analysis(Tables 16-18). The discrepancy between the number of teeth and sites was due to the presence of un-recordable information at some appointments. Previous data indicate that this does not undermine the validity of the analysis (Gilthorpe et al. 2001).

**Table 16** Baseline clinical and periodontal parameters according to patient level

| VARIABLE | PATIENTS (N=94) |
|---|---|
| Age, years | 46±9 |
| Gender, Females | 52% |
| Smokers, Current | 42% |
| BMI, $Kg/m^2$ | 25±4 |
| Ethnicity:<br>Caucasians<br>African/Caribbeans<br>Asians | <br>62<br>18<br>14 |
| Diagnosis, Chronic Periodontitis | 75% |
| FMBS | 63%±16% |
| FMPS | 58%±20% |
| Number of pockets >4mm: | 77± 23 |
| Aa detectable (patients) | 44% |
| Pg detectable (patients) | 72 % |
| Tf detectable (patients) | 76% |
| Treatment Time, minutes | 193±38 |

**Table 17** Baseline clinical and periodontal parameters according to tooth level

| VARIABLE | TOOTH (N=2589) |
|---|---|
| Tooth Type: | |
| Incisors | 550 |
| Premolars | 654 |
| Molars | 1385 |
| Mobility, Grade | |
| 0 | 1582 |
| I | 740 |
| II | 201 |
| III | 66 |
| Furcation involvements: | |
| Molars | 58% |
| Upper First Premolars | 25% |

**Table 18** Baseline clinical and periodontal parameters according to site level.

| VARIABLE | SITE (N=15498) |
|---|---|
| PPD, mm | 4.4±0.4 |
| REC, mm | 0.51± 1.70 |
| CAL, mm | 4.93±1.13 |
| Plaque Score (1) | 58.5% |
| BoP (1) | 64.1% |

Non surgical periodontal therapy using a piezoelectric device required an average of 193±38 minutes and resulted in a significant improvement in all clinical periodontal parameters after six months (Table 19). A mean reduction of 57.5±2.4 (mean±se) in the number of periodontal pockets greater than 4mm ($p<0.0001$ t-test) was achieved

together with a significant reduction in full mouth bleeding (45.5±2.5%, p<0.0001 t-test) and plaque scores (37.7±1.9%, p<0.0001 t-test).

**Table 19** Differences in patient level clinical outcomes 2 and 6 months following non-surgical periodontal therapy

| | Baseline - 2 Months | | | Baseline – 6 Months | | |
|---|---|---|---|---|---|---|
| | Mean ± SE | 95% CI | P | Mean ± SE | 95% CI | P |
| ΔFMPS | 36.9±2.1 | 32.8 - 41.1 | <0.001 | 37.7±1.9 | 34.0 - 41.4 | <0.001 |
| ΔFMBS | 46.8±1.7 | 43.4 - 50.3 | <0.001 | 45.5±2.5 | 42.1 - 49.0 | <0.001 |
| Δnppd >4 | 52.5±2.5 | 47.6 - 57.6 | <0.001 | 57.5±2.4 | 52.6 - 62.4 | <0.001 |
| ΔPPD | 1.1±0.5 | 1.0 – 1.2 | <0.001 | 1.2±0.5 | 1.1 – 1.3 | <0.001 |
| ΔCAL | 0.2±0.6 | 0.1 – 0.3 | 0.003 | 0.1±0.5 | -0.04 - 0.2 | 0.215 |

FMBS and FMPS stand for full mouth bleeding and plaque scores respectively, nppd>4 refers to the total number of periodontal pockets per patient probing more than 4 mm.

#### 4.6.3.1 Changes in PPD

Results from the variance component (null) multilevel model with ΔPPD as dependent variable without and with all explanatory covariates included are presented in table 20. In the null model variability at each individual level was obtained as a percentage of the total variability calculated adding all estimates together.

**Table 20** Multilevel Linear Regression Model estimating the relative contribution of patient, tooth and site parameters to the observed variability in PPD reduction

| | ΔPPD Baseline 6 Months | |
|---|---|---|
| | Null Model | Clinical Variables included |
| | β±SE | β±SE |
| Intercept | 1.053 ± 0.046 | 2.539± 0.442 |
| Patient | 0.177±0.029 (8.0%)* | 0.072±0.013 (-59.3%)† |
| Tooth | 0.262±0.017 (11.6%)* | 0.096±0.008 (-63.4%)† |
| Site | 1.806±0.024 (80.4%)* | 0.753±0.011 (-58.3%)† |
| -2 LL | 50312.170 | 28752.080‡ |

Percentage of variance in the dependent variable ΔPPD attributed by the multilevel model at the patient, tooth and site level. † Difference in percentage of variance in the dependent variable ΔPPD at the patient, tooth and site level when explanatory (clinical) variables were included in the model. ‡ -2LL change significant (P<0.0000001) tested by $\chi^2$.

The majority of the variance was attributed to the site level (80%), followed by the tooth (12%) and patient level (8%). On average, adding the tested variables to the model

explained 60% of the variability at all levels (Table 20). Significant better fit was obtained by entering all clinical covariates at all levels (P<0.00001 Chi-Square test). Regression estimates and significance testing for all clinical covariates were also performed (Table 21). Current smokers (p<0.001) and individuals carrying one or two copies of the rare allele at the IL-6 (-174) polymorphism (p<0.01) showed a significantly less favorable response to periodontal therapy in terms of changes in probing pocket depths. All other subject variables were not significant in the final model.

**Table 21** Multilevel Linear Regression Model assessing the significance of tested patient, tooth and site parameters in explaining the variability in observed PPD reductions

| | | ΔPPD Baseline 6 Months |
|---|---|---|
| | **Variable** | **B ± SE** |
| Patient | Age | 0.003±0.006 |
| | Gender | -0.089±0.077 |
| | Ethnicity | -0.095±0.050 |
| | Smoking | **-0.324±0.081 ‡** |
| | Diagnosis | 0.001±0.127 |
| | BMI | 0.002±0.010 |
| | FMBS | -0.001±0.003 |
| | FMPS | 0.002±0.003 |
| | NPPK | -0.001±0.002 |
| | FullMouth CAL | -0.099±0.057 |
| | Pg | 0.045±0.100 |
| | AA | 0.027±0.077 |
| | Tf | -0.017±0.091 |
| | Treatment Time | 0.001±0.001 |
| | N° Teeth Extracted | 0.010±0.020 |
| | IL-6 (-174) | **-0.226±0.090 †** |
| | TNF-A (-308) | -0.057±0.098 |
| | TLR-4 (-299) | -0.119±0.117 |
| | IL-1A, IL-1B, IL-10 | NS |
| Tooth | Mobility | **-0.069±0.022 *** |
| | Furcation | -0.029±0.038 |
| | Premolars vs Incisors | **-0.226±0.031 §** |
| | Molars vs Incisors | **-0.686±0.045 §** |
| Site | Plaque | 0.02±0.022 |
| | BoP | 0.011±0.025 |
| | Mesio-Distal | **0.428±0.023 §** |
| | Normalized baseline PPD | **0.633±0.007 §** |

* P<0.05 † P<0.01 ‡ P<0.001 § P<0.0001

At the tooth level, the presence of mobility at the baseline visit was also significantly associated with a less favorable PPD reduction (P<0.05) while the model clearly

demonstrated that anterior teeth (incisors and canines) showed the most significant decreases in PPD when compared to premolars or molars (P<0.0001). At the site level the model confirmed that normalized baseline probing depths were highly associated with changes in PPD (P<0.0001) and that mesial and distal sites were the areas where PPD changes were greater than those observed at the facial and lingual sites (P<0.0001). Analysis of residuals at each level of variability confirmed the normality assumptions (Figure 26 A-C) and the validity of the model.

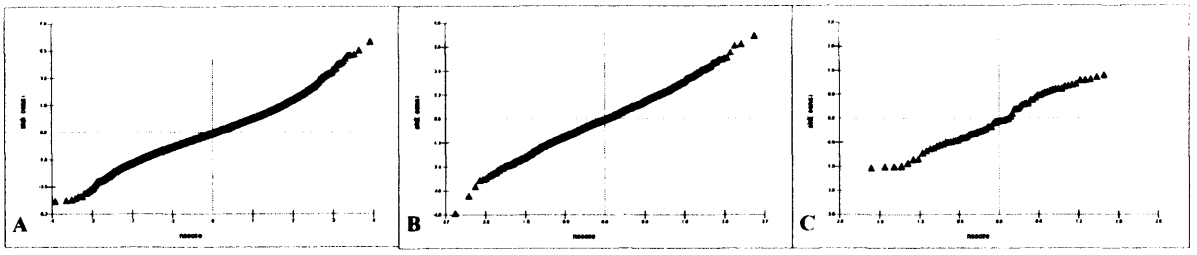


**Figure 26** Unstandardized residuals at site (A), tooth (B) and patient (C) levels of variance for ΔPPD Baseline-6 months MLM.

Table 22 shows the patient-based changes in PPD according to smoking status and carriage of the rare allele at the IL-6 gene. Within each group, PPD changes between baseline and 6 months were highly significant (P<0.001 t-test). To compare changes in PPD among groups, a standard multiple testing approach with a Bonferroni correction was utilized.

**Table 22** Patient level analysis of PPD reductions based on smoking status and carriage of the rare allele of the IL-6(-174) polymorphism (N=94)

| | VARIABLE | BASELINE MEAN ± SE | 6 MONTHS MEAN ± SE | Δ BL-6M MEAN ± SE | P T-TEST |
|---|---|---|---|---|---|
| PPD | Smoker | 4.47 ± 0.11 | 3.43 ± 0.09 | 1.04 ± 0.06 | <0.001 |
| | Non smoker | 4.30 ± 0.07 | 3.03 ± 0.05 | 1.26 ± 0.07 | <0.001 |
| nppd >4 | Smoker | 82 ± 4 | 29 ±3 | 53 ± 4* | <0.001 |
| | Non smoker | 78 ± 3 | 18 ± 2 | 60 ± 3* | <0.001 |
| PPD | IL-6 (-174) GG | 4.34 ± 0.08 | 3.13 ± 0.06 | 1.21 ± 0.06 | <0.001 |
| | IL-6 (-174) GC - CC | 4.40 ± 0.10 | 3.27 ± 0.09 | 1.13 ± 0.08 | <0.001 |
| nppd >4 | IL-6 (-174) GG | 82 ± 3 | 19 ± 2 | 63 ± 4* | <0.001 |
| | IL-6 (-174) GC - CC | 77 ± 3 | 26 ± 3 | 51 ± 3* | <0.001 |

* P<0.05 post hoc comparison (Bonferroni) Nppd>4 stands for total number of pockets greater than 4mm per patient.

This analysis showed a small but significant difference between current smokers and non smokers in terms of changes in probing pocket depths (0.22 mm 95% CI 0.03 to 0.41). The carriage of the rare allele for the IL-6 (-174) polymorphism was significantly associated with a smaller decrease in the number of pockets (mean difference 11 pockets 95% CI 2-21).

A tooth based analysis is displayed in table 23. This analysis failed to show a significant effect of tooth type (incisors vs. premolars or molars) in terms of ΔPPD.

The effect of tooth mobility, however, was highly significant: at greater baseline mobility values corresponded higher the observed decrease in PPD (P<0.001 post hoc Bonferroni comparison).

**Table 23** Tooth level analysis of PPD reductions based on tooth mobility and tooth type (N=2589)

| | | BASELINE MEAN ± SE | 6 MONTHS MEAN ± SE | Δ BL-6M MEAN ± SE | P T-TEST |
|---|---|---|---|---|---|
| Tooth Type | Molars - Premolars | 4.72 ± 0.03 (1494) | 3.49 ± 0.02 (1335) | 1.05 ± 0.02 (1330) | <0.001 |
| | Incisors | 3.86 ± 0.04 (1095) | 2.79 ± 0.02 (1066) | 1.03 ± 0.03 (1066) | <0.001 |
| | Molars | 4.60 ± 0.04 (1385) | 3.39 ± 0.03 (1230) | 1.03 ± 0.02 (1226) | <0.001 |
| | Incisors | 3.87 ± 0.05 (550) | 2.78 ± 0.03 (539) | 1.07 ± 0.04 (539) | <0.001 |
| Mobility | Grade 0 | 3.94 ± 0.03 (1582) | 3.05 ±0.02 (1525) | 0.87 ± 0.02* (1525) | <0.001 |
| | 1 | 4.71 ± 0.04 (740) | 3.32 ±0.04 (682) | 1.28 ± 0.03* (682) | <0.001 |
| | 2 | 5.63 ± 0.10 (201) | 3.82 ± 0.11 (138) | 1.58 ± 0.09* (138) | <0.001 |
| | 3 | 6.25 ± 0.17 (66) | 3.78 ± 0.20 (29) | 1.85 ± 0.20* (29) | <0.001 |

* P<0.001 post hoc comparisons (Bonferroni)

Since the majority of previous studies have been reported using flawed site-based analyses, results of this trial have also been reported using a site analysis (table 24) to allow comparison. In this instance the site based analyses were in agreement with the multilevel analysis. Mesial and distal sites showed significantly greater changes in probing depths after non surgical periodontal therapy than facial or lingual surfaces (P<0.001 t-test mean difference 0.81 mm 95% CI 0.76 to 0.86). In addition to the

previous analysis in the same table are reported the significant differences between groups of sites according to the standard cut-off values of the baseline probing depths. The deeper the baseline PPD the greater was the decrease in PPD 6 months after subgingival scaling. 1.22 mm of PPD reduction was found in sites measuring 4-5mm at baseline (P<0.001 t-test 95%CI 1.2 to 1.3); while the ΔPPD was 2.4 mm at sites with baseline PPD of 6 mm or more (P<0.001 t-test 95% CI 2.3 to 2.4).

**Table 24** Site level analysis of PPD reductions based on site location and classes of baseline PPD (N=15498)

| | BASELINE | 6 MONTHS | Δ BL-6M | P |
|---|---|---|---|---|
| VARIABLE | MEAN ± SE | MEAN ± SE | MEAN ± SE | T-TEST |
| Facial-Lingual | 3.06 ± 1.86 (5176) | 2.40 ± 1.20 (4794) | 0.51 ± 1.34 * (4786) | <0.001 |
| Mesio-Distal | 5.01 ± 1.94 (10322) | 3.57 ± 1.37 (9568) | 1.31 ± 1.50 * (9533) | <0.001 |
| PPD <=3mm | 2.27 ± 0.73 (6271) | 2.25 ± 0.80 (6064) | 0.02 ± 0.81† (6064) | <0.001 |
| PPD= 4-5mm | 4.57 ± 0.49 (4439) | 3.34 ± 1.05 (4153) | 1.22 ± 1.06† (4153) | <0.001 |
| PPD ≥6mm | 6.89 ± 1.22 (4788) | 4.39 ± 1.51 (4102) | 2.39 ± 1.52† (4102) | <0.001 |

* P<0.001 independent t-test † P<0.001 post hoc comparisons (Bonferroni) The table is derived from a flawed site-based analysis with the only purpose to compare these results with the ones derived from the multilevel modeling, rather than being interpreted on its own.

#### 4.6.3.2 CHANGES IN CAL

A Second MLM model with ΔCAL as dependent variable and including or not all explanatory covariates is presented in table 25. The null model, which represents only the intercept model, refers to the total variance of the model distributed across each level of the hierarchy (site, tooth, patient). Each level of hierarchy is highly significant and with the inclusion of all clinical parameters the difference in percentage of variance explained by the model is also reported (P<0.00001 Chi-Square test). A significant reduction in total residual variance was found at the patient and site levels whereas at the tooth level the inclusion of all clinical variables did not change significantly the

residual variation. Regression estimates and significance testing for all clinical covariate are reported in table 26.

**Table 25** MLM random intercepts for DCAL 0-6 with and without clinical variables included

| | ΔCAL BASELINE 6 MONTHS | |
|---|---|---|
| | NULL MODEL | CLINICAL VARIABLES INCLUDED |
| | β±SE | β±SE |
| Constant | -0.124±0.046 | 0.943±0.565 |
| Patient | 0.173±0.029 (7.1%)† | 0.097±0.020 (-43.9%)‡ |
| Tooth | 0.346±0.021 (14.0%)† | 0.338±0.020 (-2.3)‡ |
| Site | 1.951±0.026 (78.9%)† | 1.362±0.021 (-30.2%)‡ |
| -2 LL | 49229.620 | 34803.590§ |

† Percentage of variance in the dependent variable ΔCAL attributed by the multilevel model at the patient, tooth and site level.‡ Difference in percentage of variance in the dependent variable ΔCAL at the patient, tooth and site level when explanatory (clinical) variables were included in the model.
§ -2LL change significant (P<0.0000001) tested by $\chi^2$.

Similarly to the results for ΔPPD, smoking status and carriage of a specific polymorphism (IL-6 gene) where significantly associated with changes in clinical attachment levels after six months of periodontal therapy (P<0.001) giving strength to the overall analysis.

Subjects of different ethnicity (not Caucasian vs Caucasian) or those individuals carrying one or two copies of the rare alleles at polymorphisms of TNF-A and TLR-4 genes showed a negative association with clinical attachment level changes (table26). Furthermore the detection of a specific periodontal pathogen (*Pg*) at baseline was associated with a greater difference in CAL at 6 month follow up. At the tooth and site level similar results to the previous model (ΔPPD) were observed for all covariates included in the model. Essentially a greater difference in CAL was predicted if there was no furcation involvement at baseline (P<0.0001) or if the tooth was an incisor (P<0.001 incisors vs premolars, P<0.0001 incisors vs molars). The presence of mobility at the baseline visit was not associated with any changes in clinical attachment levels.

Finally, the model (CAL), one more time, confirmed that the baseline probing depths were highly associated not only with changes in PPD (P<0.0001) as previously reported but also with differences in clinical attachment levels (P<0.0001). Mesio-distal sites were the surfaces where these changes happened the most when compared to bucco-lingual sites (P<0.0001).

**Table 26** MLM three levels variance model for ΔCAL 0-6 with all clinical variables included

| | | ΔCAL BASELINE 6 MONTHS |
|---|---|---|
| | VARIABLE | B ± SE |
| Patient | Age | -0.003±0.008 |
| | Gender | -0.118±0.095 |
| | Ethnicity | **-0.196±0.061** † |
| | Smoking | **-0.239±0.099** ‡ |
| | Diagnosis | -0.214±0.156 |
| | BMI | -0.006±0.003 |
| | FMBS | -0.001±0.004 |
| | FMPS | **-0.006±0.003** † |
| | NPPK | -0.001±0.002 |
| | FMCAL | -0.074±0.070 |
| | Pg | **0.261±0.122** † |
| | AA | -0.019±0.094 |
| | Tf | -0.169±0.111 |
| | Treatment Time | -0.002±0.001 |
| | N° Teeth Extracted | -0.015±0.025 |
| | IL-6 (-174) | **-0.393±0.110** # |
| | TNF-A (-308) | **-0.326±0.120** § |
| | TLR-4 (-299) | **-0.286±0.143** † |
| | IL-1A- IL-B511,3954,IL-10 | NS |
| Tooth | Mobility | -0.064±0.035 |
| | Furcation | **-0.228±0.061** # |
| | Premolars vs Incisors | **-0.121±0.050** § |
| | Molars vs Incisors | **-0.207±0.072** # |
| Site | Plaque | 0.039±0.030 |
| | BoP | 0.054±0.035 |
| | Mesio-Distal | **0.561±0.122** # |
| | PPD | **0.466±0.009** # |

† P<0.05 ‡ P<0.01 § P<0.001 # P<0.0001

Confirmation of the validity of the model came from the analysis of residuals at each level of variability (site, tooth and patient) (Figure 27 A-C).

We then repeated the analysis of the above reported significant changes utilizing a standard statistical approach that does not account for the intrinsic hierarchy of periodontal data (t-test or ANOVA as appropriate). Tables 27-29 show the differences

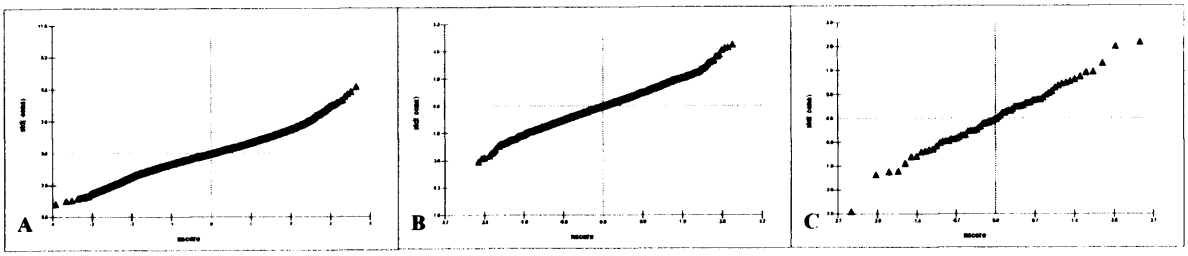


**Figure 27** Unstandardized residuals at site (A), tooth (B) and patient (C) levels of variance for ΔCAL Baseline-6 months MLM.

in terms of CAL between baseline and 6 months after periodontal therapy separately at each level of variance (patient level: table 27, tooth level: table 28 and site level: table 29).

**Table 27** Changes in CAL at patient level (mean full mouth PPD and nPPD> 4 – N=94) by: smoking, ethnicity, Pg presence and significant polymorphisms (IL-6, TNF-A, TLR-4)

| | BASELINE | 6 MONTHS | Δ CAL-6M | P |
|---|---|---|---|---|
| VARIABLES(PATIENT) | MEAN ± SD | MEAN ± SD | MEAN ± SE | |
| Smoker | 5.21 ± 0.18 | 5.25 ± 0.18 | -0.05 ± 0.08 | NS |
| Non smoker | 4.76 ± 0.15 | 4.61 ± 0.14 | 0.15 ± 0.08 | NS |
| Caucasian | 4.57 ± 0.12 | 4.60 ± 0.15 | -0.03 ± 0.10 | NS |
| Non Caucasian | 5.12 ± 0.16 | 5.00 ± 0.16 | 0.13 ± 0.07 | NS |
| Pg Positive | 5.01 ± 0.14 | 4.99 ± 0.14 | 0.02 ± 0.07 | NS |
| Pg Negative | 4.63 ± 0.25 | 4.33 ± 0.24 | 0.29 ± 0.14 | NS |
| TNF-α(-308) 1.1 | 4.76 ± 0.12 | 4.68 ± 0.13 | 0.08 ± 0.07 | NS |
| TNF-α(-308) 1.2 - 2.2 | 5.32 ± 0.32 | 5.26 ± 0.29 | 0.07 ± 0.10 | NS |
| IL-6 (-174) GG | 4.81 ± 0.13 | 4.67 ± 0.13 | 0.13 ± 0.07 | NS |
| IL-6 (-174) GC - CC | 5.10 ± 0.21 | 5.09 ± 0.21 | 0.01 ± 0.10 | NS |
| TLR-4 (-299) 1.1 | 4.85 ± 0.11 | 4.78 ± 0.12 | 0.07 ± 0.07 | NS |
| TLR-4 (-299) 1.2 - 2.2 | 5.21 ± 0.43 | 5.29 ± 0.44 | -0.07 ±0.12 | NS |

The rationale behind this supplemental analysis was to demonstrate that using a standard statistical approach some informations maybe lost and perhaps different conclusions are drawn from the analysis. Nevertheless many of the findings reported from the MLM analysis were confirmed (Table 26).

**Table 28** Changes in CAL at tooth level (mean PPD/tooth N=2589) by: tooth type (incisors vs premolars/molars) and furcation involvement (present/absent).

| | BASELINE | 6 MONTHS | Δ BL-6M | P |
|---|---|---|---|---|
| VARIABLES (TOOTH) | MEAN ± SE (N) | MEAN ± SE (N) | MEAN ± SE (N) | |
| Furcation present | 5.17 ± 0.11 (295) | 4.67 ± 0.11 (232) | 0.05 ± 0.09 (227) | <0.001 |
| Furcation absent | 5.77 ± 0.09 (537) | 5.29 ± 0.09 (466) | -0.01 ± 0.05 (460) | <0.001 |
| Molars-Premolars | 5.14 ± 0.05 (1494) | 4.87 ± 0.05 (1339) | -0.06 ± 0.03* (1327) | <0.001 |
| Incisors | 4.48 ± 0.06 (1094) | 4.56 ± 0.06 (1067) | -0.17 ± 0.03* (1065) | <0.001 |
| Molars | 5.58 ± 0.07 (840) | 5.11 ± 0.06 (705) | 0.01 ± 0.04** (694) | <0.001 |
| Incisors | 4.48 ± 0.06 (1094) | 4.56 ± 0.06 (1067) | -0.17 ± 0.03** (1065) | <0.001 |

* P<0.05 ** P<0.0001 Independent t-test

**Table 29** Changes in CAL at site level (CAL at each site N=18048) by mesio/distal vs liguo/buccal, and PPD classes at baseline

| | BASELINE | 6 MONTHS | Δ BL-6M | P |
|---|---|---|---|---|
| VARIABLES (SITE) | MEAN ± SD (N) | MEAN ± SD (N) | MEAN ± SD (N) | |
| Mesio Distal | 5.33 ± 2.57 (10086) | 5.08 ± 2.16 (9157) | 0.03 ± 1.58* (9016) | <0.001 |
| Buccal-Lingual | 3.92 ± 2.50 (5095) | 4.05 ± 2.05 (4690) | -0.39 ± 1.53* (4636) | <0.001 |
| PPD <=3mm | 2.91 ± 1.62 (6139) | 3.69 ± 1.73 (5932) | -0.82 ± 1.18** (5862) | <0.001 |
| PPD= 4-5mm | 5.00 ± 1.78 (4350) | 4.88 ± 1.85 (3950) | 0.07 ± 1.41** (3925) | <0.001 |
| PPD ≥6mm | 7.29 ± 2.25 (4682) | 6.24 ± 2.15 (3881) | 0.77 ± 1.74** (3862) | <0.001 |

### 4.6.4 DISCUSSION

The results of this analysis established that, in a group of 94 systemically healthy subjects suffering from severe, generalized periodontitis, the major source of variability in terms of decrease in probing pocket depths and CAL, following delivery of a standard phase of cause-related periodontal therapy consisting of oral hygiene instructions and mechanical subgingival debridement, was attributable to the site level (80%), with the tooth and the patient explaining 12% and 8% of residual variance. This analysis associated the extent of decrease of periodontal pockets with patient factors (cigarette smoking status and carriage of the rare allele of a specific polymorphism for the IL-6 gene), tooth factors (tooth mobility and tooth type), and site factors (mesial and distal location, as well as baseline probing depth). A multilevel analytical approach was used in order to respect the intrinsic hierarchy of periodontal clinical data (patient, tooth and site). Each level of variance was significant in the models as well as significant were a series of covariates that were used in order to predict changes in probing pocket depths and clinical attachment levels following initial periodontal therapy. These results are significant since they represent a robust confirmation and an expansion of the evidence that had been generated before a full understanding of the fact that the treatment outcomes at different sites and/or teeth within the same patient are not independent (DeRouen et al. 1991; Hujoel et al. 1990). Of interest was also the estimation of the fact that the tested clinical variables explained roughly 60% of the observed variability at the patient, the tooth and the site level. Given the fact that the present study did not involve extensive analysis of the mechanisms by which the tested therapy is likely to result in clinical improvements, the percentage of explained variability was somehow unexpected and can perhaps be better understood hypothesizing that some of the incorporated variables may represent proxy estimations of these mechanisms. As an example, PPD may in itself represent an estimation of the

level of debridement that can be achieved (Rabbani et al. 1981). This study confirmed initial observations indicating that cigarette smoking negatively affects the outcome of non-surgical periodontal therapy: smokers resulted in 0.23 mm less pocket depth reduction than non-smokers. Even though the average amount may seem small, clinicians should put this number in the context of the observed decrease for the whole population (95% CI of 1.1.to 1.3 mm). The observation that smokers had also a 0.20 mm attachment levels loss compared to non smokers further confirms the negative influence of this variable. Pocket depth reductions in smokers were decreased by 20% compared to the overall population.

A series of polymorphisms in pro-inflammatory genes were evaluated. We did not find any correlation between the IL-1A(-889) and IL-1B (+3954) on clinical periodontal parameters changes after periodontal therapy. Some evidence suggest that the combination of carriage of a copy of the rare allele in both loci is associated with a more severe periodontal infection, tooth loss, alveolar bone levels and bleeding on probing (Lang et al. 2000; Meisel et al. 2004; Meisel et al. 2003; Meisel et al. 2002; McGuire & Nunn 1999). The significant patient associated factor was the carriage of the rare allele of a polymorphism in the promoter region of the IL-6 gene.

The analysis assessed the impact of a dominant effect of this functional polymorphism. IL-6 is a key regulatory cytokine that has been implicated in the pathogenesis of periodontitis. Research from this group has discovered an association between the tested IL-6 polymorphism and the severity of periodontal destruction as well as the level of systemic inflammatory response associated with periodontitis. The present finding has not been reported before and supports the hypothesis that IL-6 is central to the homeostasis of the periodontium. People C-carriers for this polymorphism (-174) have shown also clinical periodontal infections of greater extent that those carriers of the

common allele (G) (Holla et al. 2004; Trevilatto et al. 2003). This will require confirmation in future investigations.

With respect of changes in CAL after periodontal therapy, the MLM analysis evidenced a significant negative effect of the same magnitude for TNF-A and TLR-4 polymorphisms. Both genetic variants have been preliminarily associated with a clinical periodontal phenotype of greater extent and severity perhaps because of an exaggerated inflammatory response in one case (TNF-A) or a deficient host innate response in the other (TLR-4) (Holmes et al. 2003; Tabrizi et al. 2001). Further research is however needed to ascertain whether in larger populations the same effect is repeated.

Despite the limitations arising from the qualitative nature of the microbiological analysis, the finding of the positive effect of the presence of a periodontal pathogen at baseline (*P.g*) with a greater change in attachment levels after 6 months deserve some comments. Perhaps individuals positive for this pathogen were more susceptible to a better clinical response since periodontal therapy is more likely to control the presence of this pathogen rather than *Tf* or *Aa*. The hypothesis is supported by the results that showed a greater significance in the difference of *Pg* already after two months of therapy whereas *Tf* and *Aa* where less detectable only after six months. Confirmation of these findings is however also needed and we would caution any extrapolation of these results to other populations.

It is important to note that the relatively modest contribution of patient specific variables was obtained in a population of systemically healthy subjects. It may be expected that, had this study included subjects with significant systemic conditions, more patient variability could have been expected.

At the tooth level, the impact of mobility on PPD reductions deserves some discussion. The multivariate, multilevel analysis has uncovered a negative impact of tooth mobility on PPD reductions. This result was obtained following correction for other possible

confounders included in the model such as baseline PPD. The result is in apparent conflict with the results of the univariate analysis presented in table 23 indicating that better probing depth reductions were observed in teeth with a high degree of mobility. These apparently divergent results must be carefully interpreted. The multivariate model indicates that, after correcting for possible confounders, mobility had an overall negative impact on PPD reductions. The univariate analysis, on the other hand, indicates that a mobile tooth was associated with better reductions in PPD. Since mobile teeth are likely to have lost a significant amount of clinical attachment and present with deep pockets, teeth with a higher degree of mobility are likely to display better improvements in PPD due to the deep pockets (univariate analysis). After correcting for these confounders, however, mobility in itself seemed to have a negative impact on the clinical responses following sub-gingival debridement. Multilevel modelling analysis of periodontal data has produced contrastant results difficult to interpret as for instance the protective effect of the presence of supragingival calculus in terms of long-term attachment loss (Gilthorpe et al. 2001). Further research is needed in the matter to better elucidate whether there is a biological plausibility of such results.

Of interest was also the finding that incisors and canines responded better than premolars and molars. Although no evidence is available on the subject, we hypothesize that there might be a higher efficacy of subgingival instrumentation at single rooted teeth and a higher likelihood that thinner gingival tissues associated with pockets in anterior teeth are likely to heal with more recession and hence pocket reduction than in posterior teeth. The presence of furcation involvement did not have a significant impact on PPD reduction as previously reported (Loos et al. 1988; Loos et al. 1989; Nordland et al. 1987) whereas, CAL changes were highly affected by this covariate. The interpretation of this result might have been affected by the fact that this study did not

specifically address the outcome of sites with furcation involvement (site analysis) but rather considered furcation involvement as a tooth level variable.

At the site level, greater reductions in both probing pocket depths and attachment levels were observed for interdental sites as compared to facial and/or oral. This is in agreement with previous data and consistent with the prevalent location of deeper pockets in the interproximal areas.

The impact of baseline pocket depth on the amount of pocket depth reduction was also assessed. While the periodontal literature has firmly established that deeper pockets have a greater potential for reduction, a recent investigation has warned against the effect of mathematical coupling in multivariate models incorporating baseline pocket depths as an independent variable (Tu et al. 2002). To assess the effects of this important variable and reducing the impact of mathematical coupling, baseline PPD measurements were normalized. The site analysis (table 24), agrees well in terms of magnitude with the classical studies that have indicated that the magnitude of improvement is greater in deeper pockets.

It is also noteworthy that the amounts of PPD reductions observed in the present population is in excellent agreement with those reported in previous studies and in systematic reviews of non surgical periodontal therapy. In particular, moderate pockets showed an average decrease in PPD of 1.2 mm in this study and of 1.3 mm in the summary prepared for the 1996 World Workshop(Cobb 1996); similarly deeper pockets (>5 mm) showed a 2.4 mm improvement compared to a 2.3 mm. Mean changes in CAL after therapy ranged between 0.03 mm and 0.7 of gain in sites with probing pocket depths greater than 4 and 6 mm respectively. The interest of these data lies in the fact that in this study only machine-driven instrumentation was utilized. The good agreement with the published data can be interpreted as a further indication that properly performed mechanical instrumentation can lead to excellent clinical outcomes.

In this respect it is worth noting that the total time of instrumentation required to achieve satisfaction of the clinician exceeded the 3 hours (193 minutes).

## GENERAL POINTS OF DISCUSSION

This preliminary investigation indicated that patients suffering from severe generalized periodontitis (affecting at least 50% of their dentition) also present a systemic inflammatory reaction assessed by raised IL-6 and CRP serum concentrations. The degree of this systemic reaction is positively associated with the extent and severity of periodontal infection and it is independent of age, gender, smoking and ethnical background. These data enabled us also to explore the possible influence of genetic variations on the individual chronic inflammatory response. Specific allele combinations were indeed significantly associated with a hyper-inflammatory phenotype elicited by the local periodontal infection.

Support for causality in the association between periodontitis and APR lies in the fact that standard periodontal therapy produced a significant reduction in serum inflammatory markers, which was also correlated positively with the degree of clinical response observed. This systemic anti-inflammatory effect was stable in magnitude for a period of 12 months somehow minimising the possible confounding arising from several systemic factors (age, gender, ethnicity, smoking).

The importance of a different genetic backgrounds however has to be mentioned also when we examined the individual response to periodontal therapy. A significant portion of the variability in the resulting inflammatory state following periodontal therapy was also explained by the carriage of rare alleles at polymorphic sites of pro-inflammatory genes (IL-1A, IL-6).

Within the limitation of the design of this trial we can conclude that the association between severe periodontitis and systemic inflammation might represent one of the missing mechanistic links between periodontal infections and systemic diseases such as

atherosclerosis, type-2 diabetes and adverse pregnancy outcomes. The individual basal inflammatory burden is increasingly assuming the role of predictor for future serious events (myocardial infarction, atherosclerosis, diabetes and hypertension) to the extent that new risk categories are being developed based on inflammatory markers.

In particular individuals showing on a single occasion a concentration of CRP above 3 mg/L are at high risk of developing serious coronary events in the future, especially if other risk factors are present (hyperlipidaemia, smoking, diabetes and hypertension). Individuals with more severe periodontitis have 4 times more probability to be in this high-risk category. The good news however is that if this observation is proved to be real and not just affected by a reverse causality bias (atherosclerosis, hypertension, diabetes are the cause of systemic inflammation), successful periodontal therapy might reduce the individual CRP-associated cardiovascular risk.

## CONCLUSIONS

- Severe generalized periodontitis is associated with a moderate systemic inflammatory reaction in a dose-dependent way.
- This association is modulated by well recognized cardiovascular risk factors (age, body mass index, smoking) and novel genetic variations in pro-inflammatory genes (IL-1A,IL-6, TNF-α).
- Patients suffering from severe generalized periodontitis are more likely to be at higher risk for future CRP-associated cardiovascular predicted events.
- Standard periodontal therapy decreases the individual inflammatory burden after 6 and 12 months. This reduction is greater in those who show a better clinical response. Similarly a significant reduction in CRP-associated cardiovascular risk is expected.
- Because of the cohort design of this study, these observations will need to be confirmed in randomized controlled clinical trials to minimize the risk of bias in these findings. Such trials will be described in Chapter 6 and 7.
- Specific gene polymorphisms negatively influence the clinical response of individuals receiving exclusively machine driven standard non surgical periodontal therapy.

# CHAPTER 5.

# PERIODONTAL THERAPY AS A NOVEL NON-DRUG INDUCED HUMAN MODEL TO STUDY ACUTE INFLAMMATION *IN VIVO*

### 5.1.1 SUMMARY

Chronic periodontitis and its treatment can cause a systemic inflammatory response. The aim of this study was to describe the systemic inflammatory reactions to an intensive periodontal treatment regimen. 14 otherwise healthy subjects suffering from severe chronic periodontitis were enrolled in a 1 month pilot single blind trial. Intensive periodontal treatment, consisting of full mouth subgingival root debridement delivered within a 6-hr period, was performed. Periodontal parameters were recorded before and 1 month after completion of treatment. Blood samples were taken at baseline and 1, 3, 5, 7 and 30 days after treatment. IL-1Ra, IL-6 and CRP erum concentrations were determined by ELISA. Complete blood counts were also performed. One day after treatment, mild neutrophilia and monocytosis ($P<0.05$) and lymphopenia ($P<0.01$) were accompanied by a sharp increase in inflammatory markers (IL-1Ra, IL-6, $P<0.01$). A ten-fold increase in CRP ($P<0.001$) was detected on day 1 and its kinetics followed a pattern of a classical acute phase response (significantly raised concentrations up to 1 week, $P<0.01$). 3 to 7 days after treatment, subjects presented also with a mild tendency towards a normocytic anaemic state ($P<0.01$) and a degree of lympho-thrombocytosis ($P<0.05$). The observed changes were similar to those expected following the well-characterized endotoxin-challenge model of inflammation. Intensive periodontal treatment produced an acute systemic inflammatory response of one week duration and might represent an alternative to classic endotoxin-challenge or drug-induced models to study acute inflammation in humans.

### 5.1.2 INTRODUCTION

Experimental models to study in vivo acute inflammation have been developed over the years; they utilize a variety of stimuli capable of inducing a systemic inflammatory response. Among them the endotoxin model (parenteral injection of LPS in healthy subjects) represents perhaps the most known and adopted, although the physical training model (strenuous exercise) and the vaccination model have been equally useful (Fiuza & Suffredini 2001; Martich et al. 1993; Moldoveanu et al. 2001; Parker & Watkins 2001; Shephard & Shek 1998; Shephard & Shek 1998; Shephard 2002; Suffredini et al. 1995; Hingorani et al. 2000).

In the reported models, the systemic inflammatory responses to the stimuli are measured as changes in concentrations of well-recognized serum markers (CRP, IL-6, IL-1). Some of these mediators are thought to exert direct defensive functions (like CRP) while others are involved in the amplification/regulation of the local stimulus and induction of a systemic response to it (Suffredini et al. 1999).

The concept that periodontitis and its treatment could represent a useful model to study human inflammation originates from growing evidence that periodontal infections are associated with and contribute to the systemic inflammatory burden of affected individuals. Several reports have confirmed that moderate to severe forms of periodontitis are associated with a mild systemic inflammatory response, as defined by raised serum concentrations of inflammatory markers (CRP, IL-6) (Ebersole et al. 1997; Loos et al. 2000; Noack et al. 2001; Slade et al. 2003).

Periodontitis is a prototype chronic low-grade infection. It is caused primarily by anaerobic gram negative bacteria organized in a protected biofilm in the subgingival portion of the root surface. Its treatment relies upon the mechanical removal of the biofilm and subgingival calculus deposits in order to reduce bacterial load and thus local inflammation (Williams 1990). Mechanical instrumentation of the subgingival

environment, however, results in an intense transient bacteraemia as well as significant soft tissue damage at the level of the periodontium (Lofthus et al. 1991; Waki et al. 1990). The fact that periodontitis is caused by a mixed anaerobic gram negative flora would suggest that bacteraemia following periodontal instrumentation may challenge the systemic inflammatory response in a similar fashion with respect to endotoxin injection. Since mechanical periodontal therapy in the absence of administration of antimicrobials represents the standard of care delivered to the 10-15% of the adult population affected by chronic severe periodontitis, periodontal therapy may represent a readily available experimental model to study inflammation.

Improved understanding of the early inflammatory responses following delivery of periodontal therapy is also a critical element in designing definitive intervention trials to assess the nature of the association between periodontitis and systemic inflammation. These trials are designed to uncover a decrease in inflammatory parameters after periodontal therapy and such decreases could be masked by the tail of the treatment associated increase in inflammation.

The aims of this pilot investigation were i) to describe the changes in systemic inflammatory parameters consequent to an intensive periodontal treatment regimen, and ii) to determine the kinetics of changes in inflammatory markers and mediators (CRP, IL-6, IL-1Ra) in the early days and weeks following the treatment associated bacteraemia.

### 5.1.3 METHODS

#### 5.1.3.1 STUDY POPULATION AND DESIGN

The study was a prospective single blind intervention trial with one month follow-up. Participants were recruited from subjects referred to the Department of Periodontology of the EDI, UCL. Subjects presenting with severe (probing pocket depths greater than 6 mm and marginal alveolar bone loss greater than 30%), generalized (at least 50% of teeth affected) periodontitis were invited to participate in the study. Exclusion criteria included: i) known systemic diseases, ii) history and/or presence of other infections, iii) systemic antibiotic treatment in the preceding three months, iv) treatment with any medication known to affect the serum level of inflammatory markers, v) pregnant or lactating females. All patients gave written informed consent; the study had been reviewed and approved by the Eastman/UCL Hospitals joint ethics committee.

A baseline visit was conducted by a blind calibrated examiner who collected a complete medical history and standard clinical periodontal parameters (presence of plaque, bleeding on probing, probing pocket depths, and recession of the gingival margin). Subjects thereafter received an intensive session of subgingival mechanical instrumentation under local anaesthesia (within a 6 hour period). Instrumentation was performed using a piezoelectric instrument (EMS, Nyon, Switzerland) equipped with fine tips for access in the subgingival environment. Extraction of hopeless teeth was performed during the same

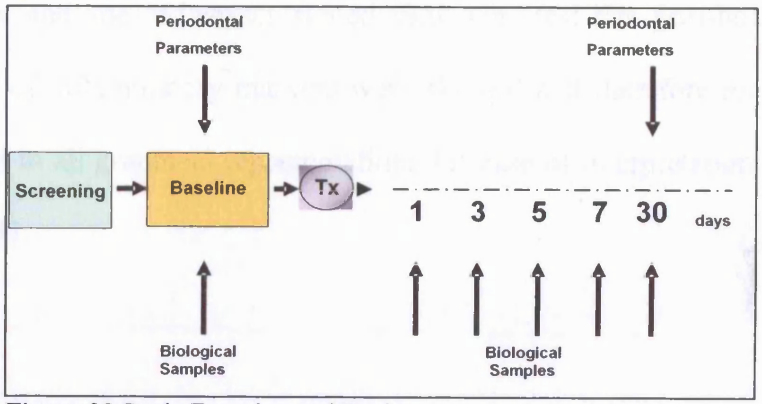


**Figure 28** Study Experimental Design

session according to standard clinical practice. Periodontal outcomes were re-assessed 1 month following completion of periodontal therapy (Figure 28).

#### 5.1.3.2 INFLAMMATORY MARKERS AND BLOOD COUNTS ANALYSES

Serial blood samples were drawn into vacutainer tubes (see section 3.4) at baseline, 1, 3, 5, 7 and 30 days after periodontal therapy. Serum was obtained by centrifugation as described earlier (section 3.4) and stored at –70°C until analysis in a standardized blind fashion. CRP levels were assessed by an automated immunoturbidimetric high-sensitivity assay (Cobas Integra, Roche AG Diagnostics, Mannheim , Germany detection limit of 0.25 mg/L) essentially as described in section 3.4.2; IL-6 and IL-1Ra were measured with high-sensitivity 2-site ELISA kits (Quantikine HS, R&D System, Minneapolis, USA, detection limit 0.04 pg/mL and 14 pg/mL respectively). Differential blood counts were performed using standard clinical haematology procedures on an automated analyzer. Body temperature was recorded at each visit using an automated thermometer (Thermoscan Plus, Ear Thermometer, Welch Allyn Braun, Thame, UK).

#### 5.1.3.3 STATISTICAL METHODS

Data are reported as means ± standard deviations (SD) for normally distributed variables or median and interquartile ranges (IQR). Non parametric statistical comparisons were applied. All statistical comparisons between visits were performed with the Friedman ANOVA and the Wilcoxon signed rank-sum test for post-hoc comparisons. Concentrations of inflammatory markers were skewed and therefore log transformed values were used in all graphical representations for ease of interpretation. The alpha value was set at 0.05.

### 5.1.4 RESULTS

14 subjects (mean age 48±6 years) were included in the trial. With the exception of the presence of severe, generalized periodontitis, all subjects were medically healthy. 8 patients were females, 10 were Caucasians, 2 subjects were smokers and 1 was a former smoker. They had an average body mass index of 26±4 kg/m$^2$. During the study period, all patients remained stable and there were no changes in lifestyle issues and habits including exercise, diet, smoking and medications.

In terms of periodontal parameters participants presented with high levels of gingival inflammation (full mouth bleeding scores of 65±14%) and severe widespread periodontitis (average of 67 ± 23 periodontal pockets 5mm or deeper per subject with an average clinical attachment level loss of 4.8 ± 0.9 mm).

**Table 30** Changes in Clinical Parameters after Periodontal Therapy

| | BASELINE | 30 DAYS |
|---|---|---|
| NPPD | 66.80±23.02 | 21.33±12.03* |
| PPD (mm) | 4.12±0.76 | 2.95±0.31* |
| REC (mm) | 0.70±0.46 | 1.30±0.51* |
| CAL (mm) | 4.82±0.87 | 4.26±0.62** |
| FMPS | 49.07±22.27 | 13.79±11.73* |
| FMBS | 65.40±14.60 | 17.29±7.96* |

Values are expressed as mean±SD, * P<0.0001, ** P<0.01 Wilcoxon post hoc rank sum paired test versus baseline.

Significant improvements in all clinical parameters were observed (P<0.0001, paired t-test) 1 month after completion of treatment (Table 30). Subgingival instrumentation was performed for an average of 200±27 minutes over a 6 hour period. A mean of 3±3 hopeless teeth were extracted per patient at the same visit. At day 1 all participants reported a series of symptoms occurring the evening after the treatment, including headache, rise in body temperature, tiredness, chills and general malaise. No significant changes were observed in participants' body temperature between the various time points (data not shown).

Box and whiskers plots of the log transformed concentrations of inflammatory markers (IL-1Ra, IL-6 and CRP) at baseline and 1, 3, 5, 7, and 30 days after treatment are displayed in figure 29. IL-1Ra kinetics showed a significant increase of its concentrations only 1 day after treatment (917.94±569.33 pg/mL compared to 572.91±248.89 pg/mL at baseline, P<0.001 Wilcoxon rank-sum test). No changes were observed at later time points for this early marker.

IL-6 concentrations were sharply increased at day 1 (6.98±1.60 pg/mL compared to 1.78±1.67 pg/mL at baseline, P<0.001 Wilcoxon rank-sum paired test) and remained higher than baseline for the first week.

Clear significant changes in CRP concentrations were observed between baseline and 1, 3, 5, 7 days after treatment (P<0.0001 Friedman ANOVA).

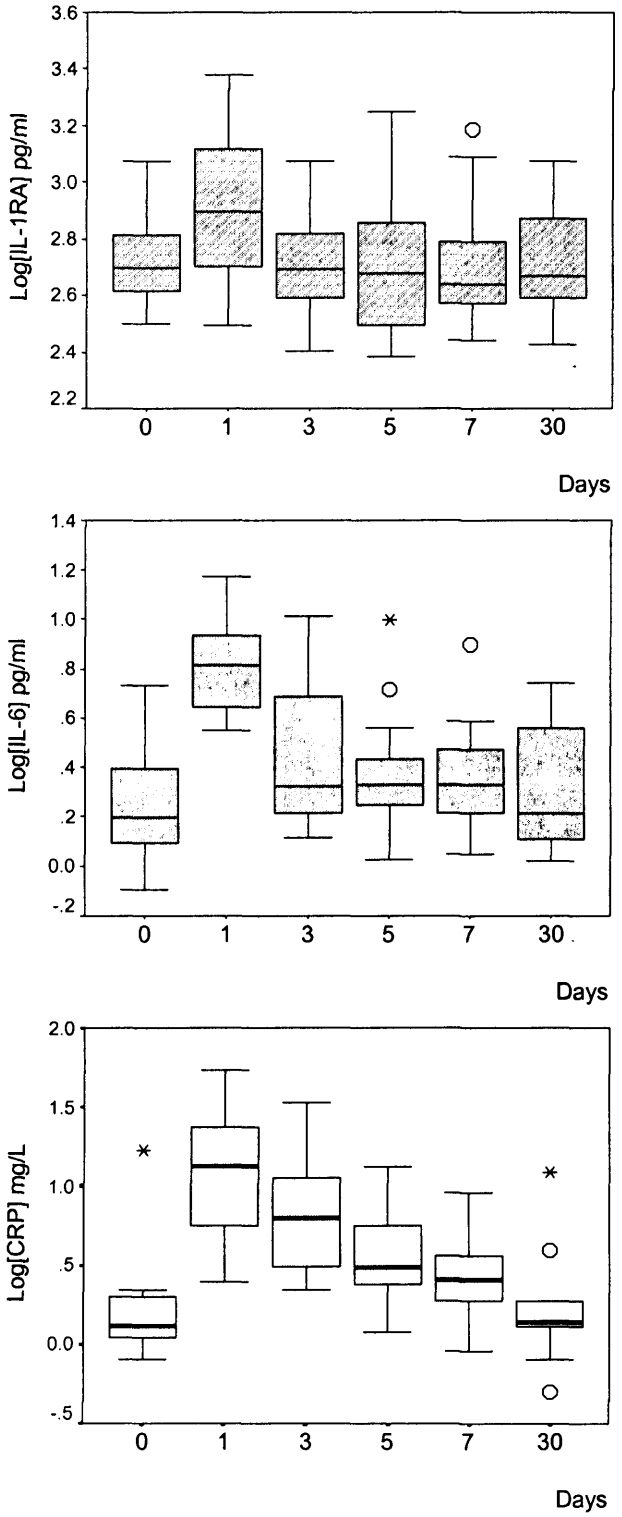


**Figure 29** Box and whiskers plots showing changes in Log[IL-1Ra], Log[IL-6], Log[CRP]before and after periodontal therapy.
The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations.

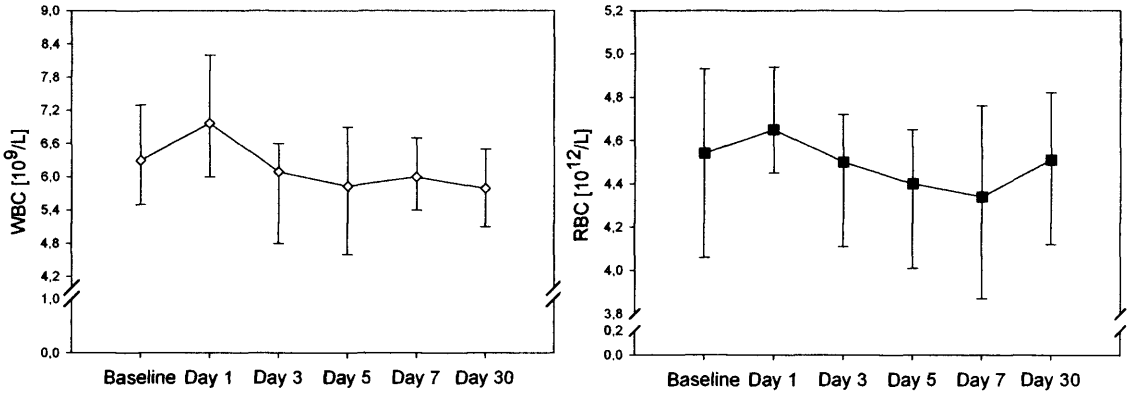


**Figure 30** Line graphs showing changes in WBC and RBC after periodontal therapy
(See table 31 for details)

From an initial 1.61±2.11 mg/L, participants showed a significant increase of CRP concentrations at day 1 of 12.49±2.63 mg/L ($P<0.0001$ Wilcoxon signed rank-sum test). CRP concentrations remained significantly higher than baseline 3, 5 and 7 days after treatment (7.31±2.40, 3.63±1.99, 2.59±1.81 respectively, $P<0.001$ Wilcoxon rank-sum paired test).

With respect to haematological parameters, early changes in differential leukocyte counts were observed at day 1.

WBC increased on day 1 after therapy even though this was not statistically significant, thereafter a significant reduction in WBC were observed after 1 month of periodontal therapy ($P<0.05$ Wilcoxon rank-sum paired test) (Figure 30).

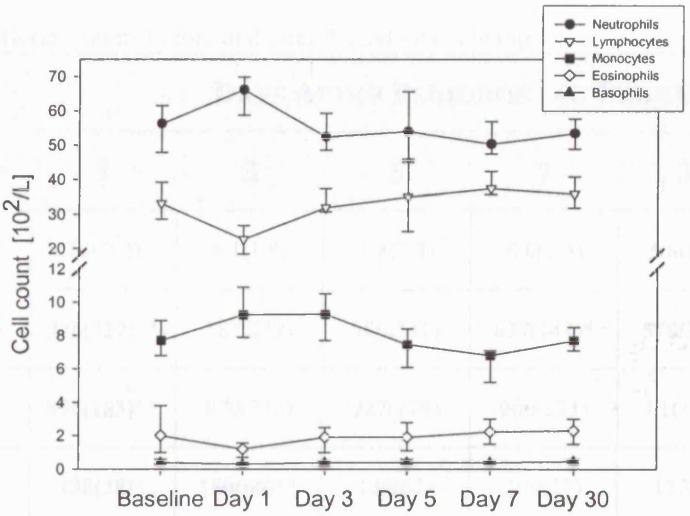


**Figure 31** Line graphs showing changes in Differential White Cell Blood Counts (See table 31 for details)

The changes at day 1 were mainly attributed to an increased pool of circulating neutrophils ($P<0.01$ Wilcoxon test compared to baseline) and an increase in the numbers of circulating monocytes was also observed at day 1 and 3 ($P<0.05$ and $P<0.01$ respectively). Lymphocyte numbers however fell ($P<0.01$) at day 1 and then increased 7 and 30 days after treatment ($P<0.05$) (Table 31 and Figure 31).

5 and 7 days after treatment, erythrocyte numbers (figure 30), haematocrit, and haemoglobin concentration decreased (Table 2). The number of platelets was significantly increased on day 5 ($P<0.05$).

**Table 31** Differential Blood Counts before and after Periodontal Therapy

| | | DAYS AFTER PERIODONTAL THERAPY | | | | | |
|---|---|---|---|---|---|---|---|
| | BASELINE | 1 | 3 | 5 | 7 | 30 | P† |
| WBC $10^9$/L | 6.3(1.8) | 7.0(2.2) | 6.1(1.8) | 5.8(2.3) | 6.0(1.3) | **5.8(1.4)*** | 0.029 |
| Lymp $10^9$/L | 554(189) | **346(222)**** | 587(279) | 586(541) | **637(242)*** | **578(354)*** | <0.0001 |
| Neutr $10^9$/L | 887(75) | **996(183)**** | 970(278) | 927(219) | 900(223) | 916(225) | 0.04 |
| Mon $10^9$/L | 114(39) | **138(28)*** | **150(66)**** | 136(62) | 109(37) | 137(37) | 0.012 |
| Baso $10^9$/L | 5(5) | **4(2)*** | 5(8) | 6(6) | 7(5) | 8(2) | 0.01 |
| Eosin $10^9$/L | 25(33) | 20(14) | 35(24) | 37(33) | 37(26) | 40(17) | 0.007 |
| RBC $10^{12}$/L | 4.5(0.9) | 4.7(0.5) | 4.5(0.6) | **4.4(0.6)*** | **4.3(0.9)**** | 4.5(0.7) | <0.0001 |
| HB g/dL | 13.7(2.5) | 14.0(1.5) | **13.5(2.7)*** | **13.4(2.1)**** | **13.3(2.5)**** | 13.8(2.8) | <0.0001 |
| MCV fl | 94.1(7.6) | 94.0(4.3) | 93.5(6.7) | 94.4(8.0) | 94.2(7.0) | 94.0(6.7) | NS |
| HCT L/L | 0.42(0.1) | 0.43(0.04) | 0.42(0.1) | **0.41(0.04)*** | **0.40(0.1)**** | 0.42(0.1) | <0.0001 |
| MCH pg | 31.6(2.5) | **30.6(2.8)**** | 30.8(3.3) | 31.0(2.5) | 31.1(3.5) | **30.3(2.8)**** | 0.033 |
| MCHC mg/dL | 33.1(1.4) | **32.3(1.0)**** | 33.0(1.5) | 32.6(1.2) | 32.8(2.0) | 37.2(1.2) | 0.08 |
| PLTS $10^9$/L | 290(85) | 289(87) | 284(78) | **309(65)*** | 291(59) | 286(53) | 0.005 |

WBC=White Cell Count, RBC=Red Cell Count, PLTS=Platelets, Lymp=Lymphocytes, Neut=Neutrophils, Mon=Monocytes, Baso=Basophils, Eosi=Eosinophils, HB=Haemoglobin, MCV=Mean Corpuscular Volume, HCT=Haematocrit, MCH= Mean Corpuscular Hemoglobin, MCHC= Mean Corpuscular Hemoglobin Concentration

† Friedman ANOVA

*P<0.05, ** P<0.01 Wilcoxon post hoc rank sum paired test versus baseline.

### 5.1.5 DISCUSSION

This report described the effects of an intensive periodontal treatment regimen (full mouth subgingival instrumentation delivered within a 6-hour period) on biochemical and haematological parameters in the days following therapy. Transient alterations in complete blood counts (mild neutrophilia and monocytosis and relative lymphopenia) were observed 1 day after treatment. 2 to 6 days thereafter a tendency towards a mild normocytic anaemic status developed. Significant changes in inflammatory markers accompanied these cellular responses: CRP, a prototype acute phase marker, increased nearly 10 fold after 1 day and returned to baseline values only 1 month after treatment. Its concentration changes followed the pattern of a classic acute phase marker with a rise within 24 hours. Data indicated that IL-6 followed the same pattern with a significant increase on day 1 and slower decreases over one week. Serum IL-1Ra levels, however, followed a different pattern. A relatively modest increase was detected only on day 1 after therapy, possibly reflecting the tail of a rapid acute response resolved within the first 24 hours.

Given the evidence of significant ingress of bacteria into the systemic circulation following periodontal instrumentation (Daly et al. 1997; Daly et al. 2001; Lofthus et al. 1991; Waki et al. 1990; Lineberger & De Marco 1973), the present data describe the systemic inflammatory changes occurring after periodontal treatment. As bactaeremia and tissue damage are produced, local and systemic production of pro-inflammatory mediators (such as TNFα and IL-1β) has been described as a component of host defences (Birkedal-Hansen 1993; Suffredini et al. 1995; Suffredini et al. 1999). In order to modulate the process, anti-inflammatory molecules (such as IL-1Ra, IL-6) appear on the scene to control inflammation and stimulate the hepatic synthesis of acute phase proteins (CRP) (Gabay & Kushner 1999). Changes in leukocytes and erythrocytes

numbers become transiently apparent in the first few days to a week (Suffredini et al. 1999).

The observed results, although different in magnitude of changes, are in agreement with those reported in several models of human inflammation. Intravenous endotoxin administration results in a systemic syndrome of several hours with fever and constitutionals symptoms. A rapid decrease in leukocyte counts due to peripheral vascular margination is observed and it is subsequently followed by a degree of neutrophilia. Production of pro-inflammatory mediators (TNF-α accompanied by modest IL-1β increase) amplify the reaction against endotoxin, while anti-inflammatory cytokines (IL-6, IL-1Ra) trigger the hepatic response that, within 24 hours, results in raised concentrations of acute phase proteins in order to contain and clear the microbial insult (Suffredini et al. 1995; Suffredini et al. 1999).

The mild tendency towards an anaemic status following intensive periodontal therapy reported in this study merits some discussion. Previous studies have indicated that following acute inflammatory stimuli (minor surgery or strenuous exercise) a post-operative/trauma anaemia develops with the characteristics of a functional iron deficiency (van Iperen et al. 1998; Fallon et al. 1999). A clear shift of the metal from the erythropoiesis and circulation to the reticulo-endothelial system might represent the pathogenetic mechanism (Weiss 2002). During a systemic inflammatory reaction (as in this and other experimental models) increased production of cytokines (IL-1, IL-6) might directly affect iron metabolism and inhibit the maturation and synthesis of the erythroid progenitor cells (Alvarez-Hernandez et al. 1989; Means, Jr. & Krantz 1992; Means, Jr. 1995; Means, Jr. 2003). Furthermore a specific IL-6-induced hepatic mediator (hepcidin) could be responsible for iron sequestration in macrophages and a reduced iron absorption in the small intestine (Ganz 2003). Patients suffering from periodontitis already manifest signs of anaemia of chronic diseases when compared with

healthy controls (Hutter et al. 2001). Further investigations are necessary to gain insight into the nature of the association between periodontitis and anaemia and the mechanisms of these observations.

Due to the lack of observations in the early post-treatment hours, this study offers no direct evidence on the peak of the inflammatory response generated by periodontal therapy. Comparisons with other inflammatory models, however, would suggest that the peak of intensity could occur within the first 2-5 hours after treatment. This interpretation is consistent with the observation that rapid inflammatory markers such as IL-1Ra had returned to baseline levels within the first 3 days. Further studies focusing on the early inflammatory responses (up to first 6-8 hrs) are needed. On the basis of these results, it is concluded that intensive periodontal treatment induced an acute systemic inflammatory response. Since the observed response seems to share many of the features of the well-characterized endotoxin, vaccination and strenuous exercise models, periodontal therapy may represent a useful non drug-induced model to study human inflammation. Its relevance may be particularly evident when researchers wish to study the functional relevance of specific genetic variants or the effect of pharmacological interventions that require relatively large sample sizes and when the use of the other established models (such as the endotoxin challenge) may be impractical or unethical.

The lack of persistence of the acute systemic inflammatory response following treatment until the first post-operative month, on the other hand, suggests that assessing inflammatory markers at later time points should not be unduly influenced by the tail end of the acute inflammatory response. This information is critical for designing the necessary randomized controlled trials to prove causality in the association between systemic inflammation and periodontitis.

# Inter-individual Variation in the Acute Release of CRP Response Following Periodontal Therapy.

## Influence of the +1444C>T Polymorphism in the CRP Gene.

### 5.2.1 SUMMARY

Above-average CRP concentrations are associated with an increased risk of future coronary events in prospective studies and it has been suggested that CRP could be used to aid risk prediction. A +1444C>T polymorphism in the CRP gene has been associated with differences in CRP concentration. We investigated the effect of this polymorphism on the CRP response to periodontal therapy, an intermediate inflammatory stimulus. Clinical parameters, CRP, and IL-6 concentrations were evaluated in 55 consecutive patients suffering from periodontitis at baseline, 1, 7 and 30 days after an intensive course of periodontal treatment. In a multivariate analysis individual homozygous for the +1444T allele showed higher CRP concentrations (day 1, 21,10±4,81 mg/L and day 7, 4,89±0,74 mg/L) compared with C-allele carriers (day1, 12,37±1,61 mg/L and day 7, 3,08±2,00 mg/L). Homozygosis for the +1444T allele of the CRP gene was associated with a greater acute CRP release after a moderate inflammatory stimulus. This effect was independent of conventional cardiovascular risk factors and inflammatory factors known to affect CRP concentrations. CRP genotype may need to be considered when CRP values are used in coronary risk prediction.

### 5.2.2 INTRODUCTION

Inflammation plays a significant role in the pathogenesis of atherosclerosis.(Libby et al. 2002) Prospective studies indicate a robust and strong association, in healthy individuals and subjects with pre-existing atherosclerosis, between levels of CRP and later cardiovascular events (Ridker et al. 1997; Ridker et al. 2000b). The CRP rise seen during acute coronary syndromes is also predictive of adverse outcome in the short term (Biasucci et al. 1999). Therefore, it has been proposed that measurement of CRP may be a useful adjunct to coronary risk assessment (Pearson et al. 2003). The sensitivity of CRP to acute intercurrent inflammatory stimuli together with its wide dynamic range (0.1-1000 mg/L) render it an excellent clinical marker of infective or inflammatory episodes but represent a challenge to its clinical use in coronary risk prediction (Pepys & Hirschfield 2003). Understanding the factors that regulate CRP release at baseline and during infection or inflammation is therefore critically important in facilitating the correct interpretation of elevated CRP concentrations in the context of risk prediction. Although CRP might have an important role in the pathogenesis and prediction of coronary events, the factors, others than IL-6, influencing the basal and stimulated CRP concentrations achieved during acute inflammation in natural occurring human models are incompletely understood. Twin and family studies indicate that CRP concentrations might be influenced by specific genetics backgrounds (MacGregor et al. 2004; Pankow et al. 2001). A polymorphism (+1444C>T) in the 3' untranslated region of the gene encoding CRP has been recently identified. In healthy subjects and CABG patients, homozygosity for the T allele of this polymorphism was associated with higher basal and stimulated CRP concentrations in male subjects (Brull et al. 2003). However, it is likely that other important genetic influences such as, the –174G>C polymorphism of the IL-6 gene (Fishman et al. 1998; Vickers et al. 2002) may also determine the basal

and stimulated concentrations of CRP in natural occurring models of chronic inflammation.

In the previous pilot trial we demonstrated how intensive periodontal therapy causes a sharp rise in inflammatory markers (CRP, IL-6) sustained up to one week from the stimulus. Periodontitis is a natural occurring prototype of chronic low-grade infection and inflammation, which has been also associated in prospective studies with an increase risk of cardiovascular events (Scannapieco et al. 2003). We therefore evaluated the effect of +1444C>T polymorphism on CRP acute release after intensive periodontal therapy and its dependence from the IL-6/–174G>C polymorphism and standard CV risk factors.

## 5.2.3 METHODS

### 5.2.3.1 Study Subjects

The study was conducted on 55 consecutive healthy individuals referred to the Department of Periodontology of the EDI, UCL. Only subjects presenting with severe (probing pocket depths greater than 6 mm and marginal alveolar bone loss greater than 30%), and generalized (at least 50% of teeth affected) periodontitis were invited to participate in the study. Exclusion criteria included known systemic diseases (hypertension, diabetes, dyslipidaemia and history of myocardial infarction or stroke), history and/or presence of other acute or chronic infections, systemic antibiotic treatment in the preceding three months, treatment with any medication known to affect the serum level of inflammatory markers (e.g. statins, steroids, hormone replacement therapy) and or pregnant or lactating females. All patients had given written informed consent; the study had been reviewed and approved by the Eastman/UCL Hospitals joint ethics committee.

### 5.2.3.2 Study Design

A baseline visit was conducted to collect a complete medical history and standard clinical periodontal parameters (PPD, REC, CAL). Subjects thereafter, received an intensive session of subgingival mechanical instrumentation (including extraction of compromised teeth) under local anaesthesia (within 6 hrs) as described in section 5.1.3.1(Figure 32). During the study period (1 month follow up), patients remained stable and there were no

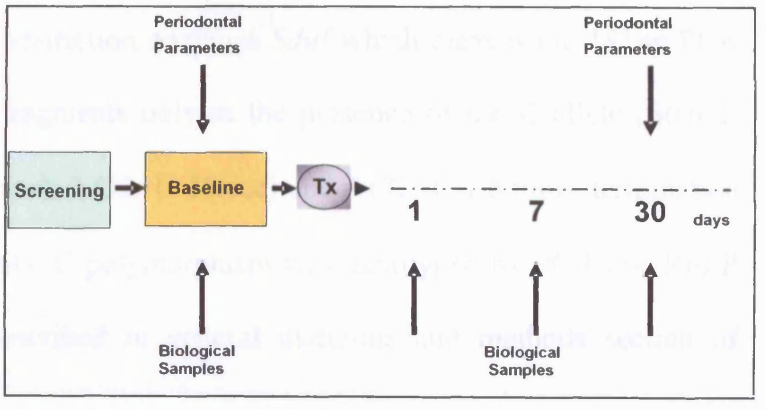


**Figure 32** Study Experimental Design

changes in lifestyle issues and habits including exercise, diet, smoking or medications when patients were asked at following visit.

#### 5.2.3.3 Inflammatory Markers Assays

Serial blood samples at baseline, one, seven and 30 days after periodontal therapy were obtained. Serum CRP concentrations were assessed by an automated immunoturbidimetric high-sensitivity assay (Cobas Integra, Roche AG Diagnostics, Mannheim, Germany) with a detection limit of 0.25 mg/L and inter, and intra-assay coefficient of variation of 4% and 5%, respectively. IL-6 was measured with high-sensitivity sandwich ELISA kits (Quantikine HS, R&D System, Minneapolis, detection limit 0.04 ng/L and inter, and intra-assay coefficient of variation less than 5%. Laboratory measurements were carried out in a blind fashion and in single batches to limit inter-assays variability.

#### 5.2.3.4 CRP and IL-6 Genotyping

DNA was extracted from patients' leucocytes as mentioned earlier in section 4.2.6. The CRP/+1444C>T polymorphism was genotyped by PCR and RFLP analysis using primer pairs ( 5'-AGCTCGTTAACTATGCTGGGGCA- 3'/5'-CTTCTCAGCTCTT-GCCTTATGAGT-3' ) and the restriction enzymes *SduI* which cleaves the 181bp PCR product into 23bp and 158bp fragments only in the presence of the C allele [Step 1, 95°C 4 min., step 2 (95°C 30 sec), 3 (55°C 30 sec) and 4 (72°C 1.5 min), termination step 72°C 3min]. The IL-6/174G>C polymorphism was genotyped by PCR and RFLP analysis using primer pairs described in general materials and methods section of chapter 4.

#### 5.2.3.5 Statistical Methods

Preliminary analysis of normality was performed using the Shapiro-Wilk test. For CRP and IL-6, because of the skewed distribution, logarithmic transformations were used and data are reported as geometric mean ± SD. Changes in serum concentrations of CRP following periodontal therapy were used as the outcome variable in a one-way ANCOVA analysis adusting for potential confounders: age, gender, ethnicity, body mass index (expressed in $kg/m^2$), smoking, blood pressure, periodontal diagnosis, number of teeth extracted and IL- 6. Furthermore a repeated measures ANOVA analysis was performed to determine also the effect of the (CRP/+1444C>T and IL-6/-174G>C) polymorphisms on time course of inflammatory markers, after adjustment for potential confounders. For these analyses recessive (CRP/+1444C>T) and dominant (IL-6/-174G>C) genetic models were used as an a priori hypothesis (Brull et al. 2003). Post hoc analyses were performed by Bonferroni corrections. A $\chi^2$ test was used to compare genotype frequencies according to the Hardy-Weinberg equilibrium. Linkage disequilibrium between CRP/+1444C>T and IL-6/-174G>C polymorphisms was estimated using the method of Chakravarti (Chakravarti et al. 1984). A *P*-value <0.05 was considered significant. Data were analyzed with the statistical software package SPSS (SPSS version 11, Chicago, IL).

## 5.2.4 RESULTS

### 5.2.4.1 DESCRIPTION OF THE STUDY SUBJECTS

Demographic and clinical characteristics of the subjects included according to the CRP/+1444C>T polymorphism are reported in table 32. With the exception of the smoking status (p=0.02), no significant differences for any other traditional cardiovascular risk factors or dental parameters including severity and extent of periodontal infections were observed at baseline according the CRP 1444C>T polymorphism.

**Table 32** Baseline characteristics of the subjects included according to the CRP/+1444C>T genotype.

| VARIABLE | OVERALL (N=55) MEAN±SD (95% CI) | C-CARRIERS (N=45) MEAN±SD (95% CI) | TT (N=10) MEAN±SD (95% CI) | P-VALUE † |
|---|---|---|---|---|
| Age, years | 48±7 (46.4-50.1) | 48±7 (45.6-49.6) | 51±7 (46.5-56.1) | 0.12 |
| Gender, Males (%) | 29(52.7) | 24(53.3) | 5(50.0) | 0.85* |
| Ethnicity, Caucasians (%) | 40(72.7) | 34(75.6) | 6(60.0) | 0.43* |
| BMI, $Kg/m^2$ | 26±4 (24.7-26.8) | 26±4 (24.5-27.1) | 26±1 (24.6-26.1) | 0.89 |
| Systolic BP, mm Hg | 136±16 (131.2-140.1) | 134±17 (129.2-139.3) | 142±14 (131.7-152.1) | 0.20 |
| Diastolic BP, mm Hg | 87±10 (79.1-93.9) | 87±11 (84.0-90.4) | 87±10 (79.1-93.9) | 0.84 |
| Smoking | | | | |
| Never (%) | 24(43.6) | 16(35.6) | 8(80.0) | 0.02* |
| Current & former (%) | 31(56.4) | 29(64.4) | 2(20.0) | |
| FMBS | 67±14 (62.7-70.4) | 65±14 (61.1-69.6) | 72±14 (62.0-82.2) | 0.17 |
| N° Periodontal lesions | 76±26 (68.7-82.7) | 74±28 (65.7-82.3) | 83±15 (72.9-93.9) | 0.30 |
| IL-6 (ng/L) | 1.51±1,88 (1.27-1.79) | 1.52±1.94 (1,25-1.86) | 1.45±1.65 (1.01-2.08) | 0.83 |
| Peak (Day 1) IL-6(ng/L) | 5.44±1.69 (4.71-6.27) | 5.27±1.70 (4.50-6.18) | 6.24±1.68 (4.30-9.06) | 0.36 |
| CRP (mg/L) | 1.93±1.95 (1.61-2.31) | 1.91±1.89 (1.58-2.31) | 2.00±2.34 (1.09-3.68) | 0.84 |

†; Between C-carriers and TT subjects. For continuous variable, a t-test was performed, and for dichotomy variables Chi-Square Test (*) was used. BMI indicates body mass index, BP blood pressure, FMBS percentage of tooth sites with bleeding upon probing.

### 5.2.4.2 Alleles and Genotype frequencies

Of the 55 evaluated subjects, 23 were homozygous for the +1444C allele, 22 were heterozygous and 10 were homozygous for the +1444T allele. Thirty individuals were homozygous for the -174G allele, 10 were heterozygous and 6 were homozygous for the -174C allele. The genotype frequencies for the +1444C>T and for the –174G>C polymorphisms were as predicted by Hardy-Weinberg equilibrium (p=0.25 and 0.27; respectively). The pairwise linkage disequilibrium coefficients (Δ) revelaed a non-significant allelic association between these two variants (Δ=0.04, p=0.76).

### 5.2.4.3 Acute phase response and effect of the +1444C>T polymorphism

The inflammatory stimulus (intensive periodontal therapy) resulted in a sharp significant increase of CRP at day one (13.64 ± 2.14 mg/L vs 1.93 ± 1.95 mg/L, P<0.0001) and day seven (3.35 ± 1.77 mg/L, P<0.0001) compared to baseline (Figure 33). These results remained statistically significant in the multivariate analysis (P<0.0001) after adjusting for age, gender, ethnicity, body mass index, smoking, blood pressure, Log[IL-6] and number of teeth extracted during treatment; (corrected model

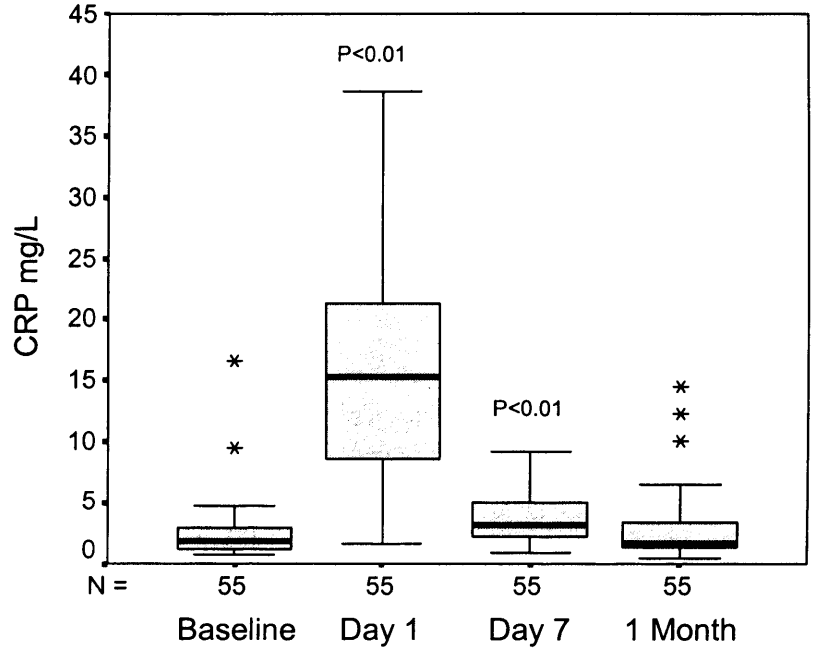


**Figure 33** Box and whiskers plots showing changes in CRP before and after periodontal therapy.
The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations

$R^2$=0.68). The magnitude of the acute persistent release of CRP was dependent of the +1444C>T genotype (Figure 34-A).

Subjects homozygous for T allele had a significantly higher concentration of CRP at day one (21.10 ± 4.81 mg/L vs. 12.37 ± 1.61 mg/L, P=0.02) and day seven (4.89 ± 0.74 mg/L vs. 3.08 ± 2.00 mg/L, P<0.01) compared to carriers of the +1444C-allele. The effect persisted (P=0.004) in the multivariate repeated measure analysis after adding all CV risk factors and the baseline and peak IL-6 concentrations.

In addition to evaluate the likelihood of confounding of the CRP-genotype and CRP acute release association by the IL-6/-174G>C variant, a separate analysis was conducted showing no significant differences in CRP acute release among individuals

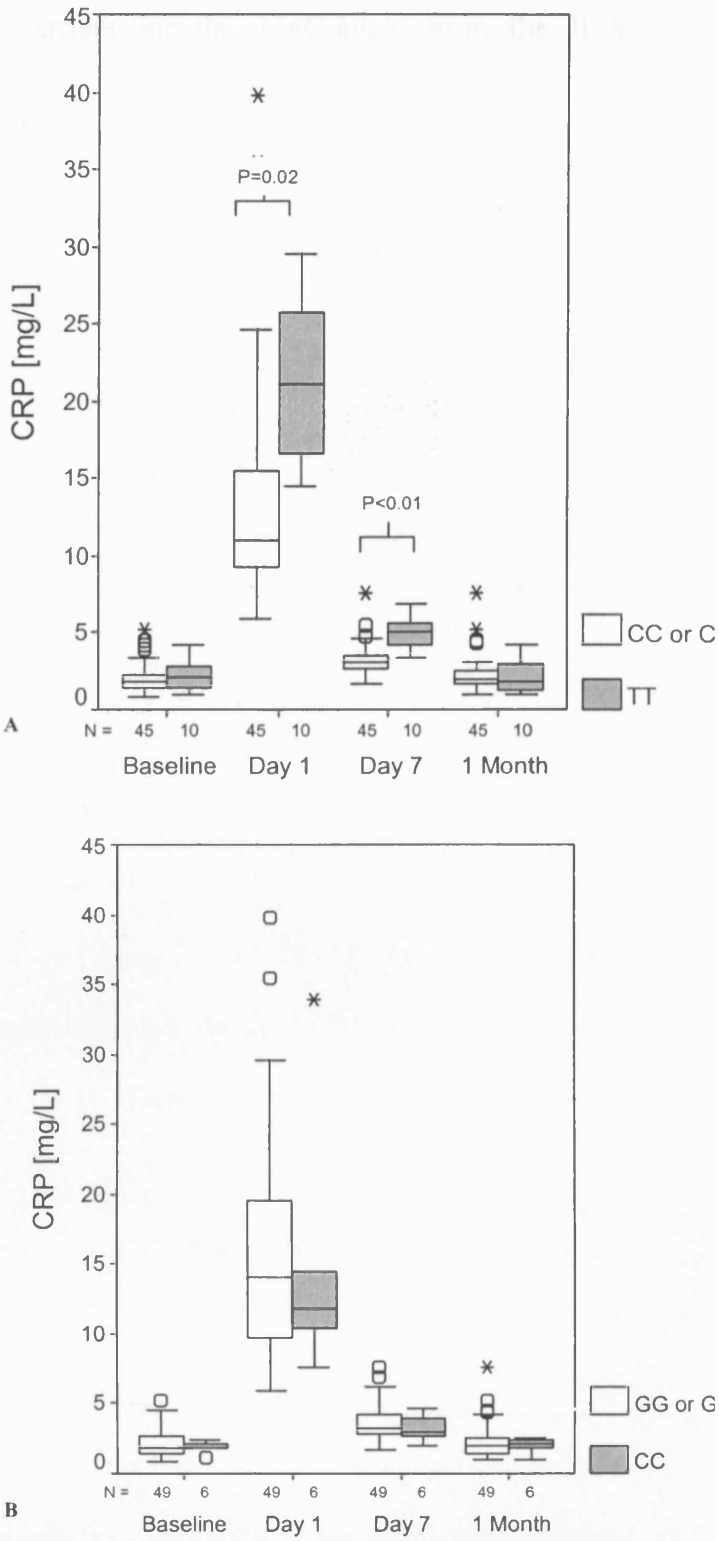


**Figure 34** Box and whiskers plots showing changes in CRP before and after periodontal therapy according to +1444C>T (A) and -174G>C (B) polymorphism's genotypes.
The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations.

homozygous for the 174G-allele and carriers for the 174C-allele from the IL-6 polymorphism. (Figure 34-B).

### 5.2.5 DISCUSSION

The main finding of this study is that, in subjects undergoing periodontal treatment homozygosis for the +1444T allele of the CRP gene was significantly associated with a greater acute CRP release after an inflammatory stimulus. This effect was independent of IL-6 concentrations, IL-6/-174G>C variant and also from conventional cardiovascular risk factors known to affect the CRP concentrations. In previous studies it has been shown that the +1444C>T polymorphism influences basal CRP, as well as the magnitude of the CRP response both to a low-grade inflammatory stimulus (48h intensive exercise; peak CRP ~ 3mg/L), as well as a more severe inflammatory stimulus (coronary artery by-pass surgery; peak CRP ~ 300mg/L)(Brull et al. 2003). Although the current study was too small to show the difference in CRP by genotype at baseline, it extends the observation that genotype modulates the response to an inflammatory stimulus. In the current study, CRP genotype was an independent predictor of the CRP response to intensive periodontal instrumentation that elevated CRP to an intermediate level (~30mg/L), confirming that over a wide range of concentrations seen in both health and disease, CRP is subject to genetic modulation. Much larger studies will now be necessary to define the precise magnitude of the genetic and environmental effects and the interaction, if any, between the two.

The observation that circulating CRP concentration is subject to genetic regulation has important implications for understanding the role of CRP in the pathogenesis of coronary artery disease and its potential role in coronary risk prediction. First, since CRP concentrations are strongly correlated with smoking status, blood pressure, obesity, diabetes, physical activity, social class, low birth weight and other products of the inflammatory response, and are also higher in individuals with clinical cardiovascular disease, the association of CRP with coronary events in observational

studies could be subject to residual confounding or, in prevalent case-control studies, to reverse causality bias. If CRP actually increases the risk of coronary events, then carriage of an allele that exposes individuals to a long-term elevation in CRP should confer an increased risk of coronary events proportional to the difference in CRP attributable to the allele. Since the inheritance of such a variant should be subject to the random assortment of maternal and paternal alleles at the time of gamete formation, according to Mendel's second law, this relationship should be largely unconfounded and free of reverse causality bias. If non-genetic observational studies of CRP are unbiased, the increase in risk estimated from these studies should be consistent with the increase in risk conferred by carriage of the allele. This approach, known as "Mendelian randomisation", has been used recently to clarify the link between homocysteine and cardiovascular disease (Smith & Ebrahim 2004; Wald et al. 2002). The identification of a common polymorphism in the CRP gene reliably associated with differences in circulating CRP concentration will allow large-scale genetic association studies to test whether the risk of coronary events in individuals homozygous for this variant is close to that predicted from non-genetic studies.

Second, if CRP is a marker for, rather than a mediator of atherosclerosis, then the prognostic value of a particular CRP level may differ by genotype. A given level of CRP may have a different implication for future risk in individuals of differing CRP genotype because it reflects differing degrees of inflammatory disease in the two cases. If this is the case, there may eventually be a need to establish genotype-specific risk thresholds for coronary risk prediction. Nevertheless, in order to evaluate this negative interaction, subjects with high-CRP due to the genotype have a lower risk than those with high-CRP without the genotype, adequately powered genetic association studies or the pooling of smaller studies are required.

## CONCLUSIONS

- Periodontal therapy represent a moderate inflammatory stimulus and it is accompanied by systemic sequelae resolving within one week from the treatment and showing similarities to those of a classical APR.
- CRP and IL-6 represent reliable inflammatory markers to study the individual basal and stimulated individual inflammatory burden.
- The acute inflammatory response associated with the delivery of periodontal therapy subsides between 1 week and 1 month after treatment. Assessment of changes in inflammatory parameters can be done at 1 month after therapy.
- Inter-individual variations exist after similar inflammatory stimuli.
- A novel polymorphism (+1444) was associated with a greater acute release of CRP independent from traditional cardiovascular risk factors and IL-6 concentrations and gene polymorphism (-174).

# CHAPTER 6.

# SHORT TERM EFFECTS OF PERIODONTAL THERAPY ON MARKERS OF SYSTEMIC INFLAMMATION

# A RANDOMIZED CONTROLLED CLINICAL TRIAL

## 6.1 SUMMARY

Severe periodontitis has been associated with increased systemic inflammation in otherwise healthy subjects. This study investigated the impact of periodontal therapy on serum inflammatory markers and cholesterol levels. 65 healthy subjects with severe generalized periodontitis participated in a single-blind, 3 arms randomized controlled clinical trial. Medical and periodontal parameters, CRP (primary outcome variable), IL-6, total cholesterol and LDL-cholesterol were evaluated prior to and two months after treatment. The 3 treatment groups included an untreated control, standard periodontal therapy (SPT) and an intensive course of periodontal treatment (IPT). 2 months after treatment, both treatment groups resulted in a significant (SPT P=0.030, IPT P=0.001) reduction of 0.5±0.2 mg/L and 0.8±0.2 mg/L respectively in serum CRP compared to the untreated control. These reductions were independent of age, gender, body mass index, ethnicity, whereas a significant interaction effect of cigarette of smoking was found. Both reduction in CRP and IL-6 were significant only in non smoker individuals (P=0.028 and P=0.003 univariate tests respectively) while in current smokers only IPT group showed a significant effect on inflammatory markers. The IPT group further showed a decrease in total cholesterol and LDL-cholesterol concentrations after two months. These data indicated that periodontitis significantly contributed to the inflammatory burden of an otherwise healthy subject.

## 6.2 INTRODUCTION

Despite the recent advances in the understanding of the atherosclerotic process, its primary cause remains unclear in the majority of cases. The presence of well defined risk factors (diabetes mellitus, hypertension, hyperlipedaemia, tobacco use and positive family history) accounts for only half of the atherosclerotic complications on the cardiovascular system (Stamler et al. 1986).

Inflammation plays a significant role in the development and progression of atherosclerosis (Libby et al. 2002). Chronic low-grade inflammation, measured as elevated CRP serum levels, has been directly associated with the onset and progression of CVD (Pearson et al. 2003). CRP hepatic production is usually elicited by an inflammatory stimulus and mediated through a complex network of cytokines (mainly IL-6); nonetheless several systemic co-factors can influence its concentration (Kluft & de Maat 2001). It is still unclear, however, whether its predictive role has etiologic implications or whether elevated CRP concentrations are only a marker of atherosclerosis and/or vascular damage. Epidemiological evidence that other inflammatory markers share the same predictive value of CRP gives strength to the significance of systemic inflammation, rather than one specific marker, on the atherosclerotic process (Pradhan et al. 2002).

A variety of sources for the atherosclerotic inflammatory process, including infectious agents, have been proposed (Danesh 1999; Lowe 2001). Periodontitis is a prototypic chronic low-grade infection. It is caused primarily by anaerobic gram negative bacteria organized in a protected biofilm in the subgingival portion of the tooth surface. It leads to inflammatory destruction of the periodontal tissues and eventually to tooth exfoliation (Williams 1990). 10-15% of adults suffer from severe forms of this disease (Albandar & Rams 2002).

Over the last 50 years, the prevailing view among dentists and physicians was that periodontal infections were localized only to the marginal periodontium and that, as such, they rarely had systemic implications in healthy individuals. More recent evidence, however, has indicated that patients with severe generalized periodontitis present with increased systemic inflammation as indicated by increased serum levels of CRP, fibrinogen, IL-6, moderate leukocytosis function when compared with unaffected control populations (Ebersole et al. 1997; Kweider et al. 1993; Loos et al. 2000; Noack et al. 2001). Further these individuals have a perturbed metabolic profile (serum cholesterol) (Katz et al. 2002) not explained just by their lifestyle but perhaps causally related to the chronic episodes of bacteraemia and endotoxin dissemination (Iacopino & Cutler 2000).

Our preliminary pilot intervention study (Chapter 4) showed how standard periodontal therapy led to a reduction in serum CRP and IL-6. The degree of observed reduction in systemic inflammation was associated with the level of dental clinical response as determined by periodontal parameters. This suggested a potential dose response between the extent of resolution of the local periodontal infection and the level of reduction in systemic inflammation. However, the limitations of the design (uncontrolled) adopted did not facilitate a proper interpretation of the results which might had been confounded by changes in other factors (body weight, smoking, medications). Standard periodontal therapy exhibited a delayed systemic anti-inflammatory effect after six months of its completion. Recent preliminary reports suggest that different treatment approaches might exert significant systemic effects (Iwamoto et al. 2003).

The specific aim of this 3-arm, single-blind, randomized, controlled intervention trial was to test the short term effects of two regimens of periodontal therapy on the systemic inflammatory status of medically healthy individuals suffering from severe,

generalized periodontitis. Changes in serum CRP levels were selected as the primary outcome variable, whereas changes in serum IL-6 and cholesterol levels were chosen as secondary outcomes.

## 6.3 METHODS

### 6.3.1 STUDY POPULATION AND DESIGN

Participants were recruited from subjects referred to the Department of Periodontology of the EDI, UCL. Only subjects presenting with severe (probing pocket depths greater than 6 mm and marginal alveolar bone loss greater than 30%), generalized (at least 50% of teeth affected) periodontitis were invited to participate in the study to increase probability of detection of a systemic burden from the local periodontal infection (Slade et al. 2000; Slade et al. 2003). Exclusion criteria included: i) known systemic diseases, ii) history and/or presence of other infections, iii) systemic antibiotic treatment in the preceding three months, iv) treatment with any medication known to affect the serum level of inflammatory markers or lipids, v) pregnant or lactating females, vi) allergy to tetracyclines. All patients gave written informed consent; the study had been reviewed and approved by the Eastman/UCL Hospitals joint ethics committee.

A baseline visit was conducted by a blind calibrated examiner who collected a complete medical history, standard clinical periodontal parameters and blood samples. Subjects were randomized by the trial coordinator, to one of the three treatments groups using a random permuted block approach and stratified based on smoking status (current vs non smoker) (Pocock 1977; Pocock 1979). Allocation was concealed using opaque envelopes which were opened by the therapist at the treatment visit. All clinical and laboratory assessments were performed blind by the investigators. A total of 70 individuals met the inclusion criteria. 65 subjects were enrolled and randomized to treatment, the remaining five subjects did not consent to the proposed plan of therapy (which included extraction of compromised teeth)(Figure 35).

The 3 groups consisted of an untreated control (which received the necessary care a the end of the trial); a community based standard regimen of periodontal therapy (SPT) consisting of subgingival mechanical instrumentation; and an intensive course of periodontal treatment (IPT) consisting of subgingival mechanical instrumentation with adjunctive local delivery of minocycline-HCl (encapsulated in PLGA microspheres for controlled delivery, Arestin®, Orapharma, Warminster, Pennsylvania, U.S.A.) into the periodontal pockets. Periodontal and inflammatory outcomes were assessed at baseline and at 2 months following completion of periodontal therapy.

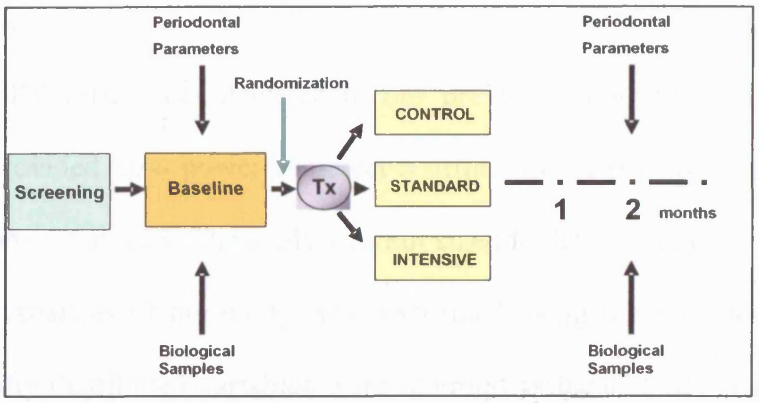


**Figure 35** Study Experimental Design

All 65 individuals reached this follow-up visit.

### 6.3.2 Inflammatory Markers and Blood Lipids Measurements

Serum samples, collected at baseline and 2 months, were processed and stored at –70°C until analysis in a standardized blind fashion. CRP levels were assessed by an automated immunoturbidimetric high-sensitivity assay (Cobas Integra, Roche AG Diagnostics, Mannheim, Germany detection limit of 0.25 mg/L); IL-6 was measured with a high-sensitivity sandwich ELISA (Quantikine HS, R&D System, Minneapolis, U.S.A., detection limit 0.04 ng/L). Total and LDL-cholesterol levels were determined using standard clinical chemistry procedures on an automated analyzer (Hitachi 917, Roche AG Diagnostics, Mannheim, Germany).

### 6.3.3 Statistical Methods

Based on the variance of CRP differences detected in our previous pilot study, 20 patients per treatment arm provided 80% power to detect a difference of 0.4 mg/L in CRP concentrations, with alpha set at 0.05. The study was not sized to detect changes in IL-6 and cholesterol levels. Analysis of normality was performed using the Shapiro-Wilk test. Continuous normally distributed variables were reported as mean ± SD and 95%CI, whereas median and IQR were used to describe not normally distributed data (IL-6 only). Significant changes in serum concentrations between baseline and 2 months of CRP were used as primary outcomes. One-way analysis of covariance was used to compare changes between 0 and 2 months in CRP serum concentrations between the three groups. Baseline levels were used as covariate. If the F statistics for the treatment effect was significant at the univariate level, multiple comparisons were performed with Bonferroni adjustment. A second model was then created including as covariates age, gender, ethnicity, body mass index and cigarette smoking.

We also examined whether IL-6, main inducer of CRP hepatic production, was affected by periodontal therapy with the same analytical approach. Changes in Total, LDL and HDL Cholesterol and Triglycerides were examined as secondary outcomes. Analysis of residuals was performed to confirm normality assumptions and validity of each model created.

Comparisons of categorical data and proportions were analyzed with the Chi-Square test. The alpha value was set at 0.05. SPSS version 11 was used (Chicago, IL, USA).

## 6.4 RESULTS

With the exception of the presence of severe, generalized periodontitis, all subjects presented with no reported medical conditions. The baseline demographic and clinical characteristics are reported in table 33. There were no significant differences between groups for any of the parameters.

**Table 33** Clinical and Periodontal Data in the 3 Groups of Patients

| VARIABLE = MEAN±SD (95% CI) | CONTROL (N=24) | STANDARD TX (N=21) | INTENSIVE TX (N=20) | SIGNIFICANCE (ANOVA) |
|---|---|---|---|---|
| Age, y | 48±6 (46-51) | 48±7 (44-51) | 49±7 (45-52) | P=0.93 |
| Females, % | 37.5 | 47.6 | 40 | P=0.78[a] |
| Caucasians, % | 66.7 | 66.7 | 85.0 | P=0.31[a] |
| BMI, $Kg/m^2$ [b] | 25.3±3.4 (23.8-26.7) | 25.7±3.5 (24.1-27.3) | 25.6±4.1 (23.7-27.5) | P=0.91 |
| Current smokers,% | 29.2 | 28.6 | 25.0 | P=0.95[a] |
| Former smokers, % | 41.7 | 52.4 | 45.0 | P=0.77[a] |
| CRP, mgL | 2.4±1.6 (1.8-3.1) | 2.9±2.2 (2.0-3.9) | 2.0±1.1 (1.6-2.6) | P=0.30 |
| Total cholesterol, mmol/L | 5.3±0.7 (5.1-5.7) | 5.3±0.7 (5.0-5.6) | 5.4±0.7 (5.1-5.8) | P=0.80 |
| LDL cholesterol, mmol/L | 3.2±0.6 (3.0-3.5) | 3.2±0.6 (3.1-3.8) | 3.4±0.6 (2.9-3.6) | P=0.64 |
| HDL cholesterol, mmol/L | 1.3±0.5 (1.1-1.5) | 1.3±0.5 (1.1-1.5) | 1.5±0.5 (1.2-1.7) | P=0.59 |
| Triglycerides, mmol/L | 1.7±1.2 (1.1-2.2) | 1.7±1.1 (1.2-2.2) | 1.4±1.1 (0.9-1.9) | P=0.67 |

[a] Chi-Square Test. [b] BMI indicates body mass index. [c] FMPS is the percentage of tooth sites with dental plaque. [d] FMBS is the percentage of tooth sites with bleeding upon probing. [e] PPD is the full mouth average periodontal pocket depth per patient expressed in mm. [f] CAL is the full mouth average clinical attachment loss per patient expressed in mm.

Subjects were not taking any anti-inflammatory, vasoactive, lipid lowering agents or systemic antibiotics. During the study period, patients remained stable and there were no changes in lifestyle issues and habits including exercise, diet, smoking and medications. Furthermore, no significant changes in body-mass index were observed for any group [mean differences ± SD of BMI of 0.1±0.7 (95% CI -0.1 to 0.4), 0.0±0.8 (95% CI -0.4 to 0.4), and 0.3±0.7 $kg/m^2$ (95% CI 0.0 to 0.6) for control (N=24), SPT

(N=21) and IPT (N=20), respectively, P>0.56]. In terms of periodontal parameters, patients had an average full mouth plaque score of 58 ± 22 % (N=65). They presented with high levels of gingival inflammation (full mouth bleeding scores of 68% ± 14%, N=65) and severe widespread periodontitis (average of 81 ± 26 periodontal lesions per subject with an average clinical attachment level loss of 5.4 ± 1.4 mm, N=65). Both SPT and IPT resulted in significant improvements of clinical periodontal parameters after two months. A mean reduction of 60±27 (P<0.0001 t-test, N=21) and 60±23 (P<0.0001 t-test, N=20) periodontal lesions were observed in the SPT and IPT groups respectively. No significant changes were observed in the untreated controls (Table 34).

**Table 34** Clinical Periodontal Parameters

| VARIABLE= MEAN±SD, | BASELINE | | | 2 MONTHS | | |
|---|---|---|---|---|---|---|
| | CTRL | SPT | IPT | CTRL | SPT | IPT |
| PPD | 4,5±0,8 | 4,3±0,8 | 4,5±0,8 | 4,4±0,7 | 2,7±0,3 | 3,0±0,3 |
| REC | 1,1±1,1 | 0,8±0,6 | 0,9±0,9 | 0,9±1,0 | 1,4±0,9 | 1,6±1,1 |
| CAL | 5,6±1,5 | 5,2±1,4 | 5,4±1,5 | 5,3±0,8 | 4,1±1,1 | 4,6±1,2 |
| FMPS | 58,4±19,2 | 59,7±20,0 | 52,1±23,2 | 56±18,3 | 11,8±7,0 | 8,2±4,6 |
| FMBS | 68,2±15,3 | 68,2±15,6 | 66,5±13,1 | 67,4±16,2 | 24,2±10,2 | 22,4±6,1 |
| NPPK | 80±26 | 79±28 | 80±25 | 79 27 | 19±4 | 20±13 |

Table 35 displays the concentrations of primary (CRP), secondary (IL-6) outcomes and lipid markers at baseline and at 2 months.

**Table 35** Concentrations of serum inflammatory parameters and lipids before and after periodontal therapy

| VARIABLE= MEAN±SD, (95%CI) | CONTROL (N=24) | | STANDARD TX (N=21) | | INTENSIVE TX (N=20) | |
|---|---|---|---|---|---|---|
| | BASELINE | 2 MONTHS | BASELINE | 2 MONTHS | BASELINE | 2 MONTHS |
| CRP mg/L | 2.4±1.6 (1.8-3.1) | 2.5±1.7 (1.8-3.2) | 2.9±2.2 (2.0-4.0) | 2.9±2.3 (1.8-4.0) | 2.0±1.1 (1.5-2.5) | 1.6±0.9 (1.2-2.0) |
| IL-6 ng/L [a] | 1.7 (1.4) (1.5-2.3) | 1.7 (0.6) (1.5-1.8) | 1.7 (1.8) (1.5-2.6) | 1.8 (1.4) (1.5-2.4) | 1.3 (0.7) (1.0-1.9) | 0.9 (0.6) (0.8-1.3) |
| Total cholesterol mmol/L | 5.4±0.7 (5.1-5.7) | 5.3±0.8 (4.9-5.7) | 5.3±0.7 (5.0-5.6) | 5.4±0.9 (4.9-5.7) | 5.5±0.7 (5.2-5.9) | 5.2±0.7 (5.0-5.5) |
| LDL cholesterol mmol/L | 3.2±0.6 (3.0-3.5) | 3.2±0.7 (2.9-3.7) | 3.2±0.6 (3.0-3.8) | 3.4±0.9 (2.9-3.7) | 3.4±0.6 (2.9-3.6) | 3.2±0.6 (3.0-3.5) |
| HDL cholesterol mmol/L | 1.3±0.5 (1.1-1.5) | 1.3±0.4 (1.1-1.5) | 1.3±0.5 (1.1-1.5) | 1.4±0.5 (1.1-1.6) | 1.5±0.5 (1.2-1.7) | 1.4±0.4 (1.2-2.6) |
| Triglycerides mmol/L | 1.7±1.2 (1.1-2.2) | 1.4±0.9 (1.0-1.8) | 1.7±1.1 (1.2-2.2) | 1.6±1.0 (1.1-2.0) | 1.4±1.1 (0.9-2.0) | 1.3±0.8 (0.9-1.7) |

[a] Median (IQR).

The covariance analysis showed a significant univariate effect of the treatment (P=0.003, Model $R^2$=0.86). A significant reduction of CRP concentrations was observed in both treatment groups when compared to the untreated control. SPT group showed a difference in CRP at 2 months of 0.5±0.2 mg/L (95% CI 0-0.9, P=0.030 Bonferroni corrections)(Figure 36) compared to the untreated control group. The respective value for IPT group patients was 0.8±0.2 mg/L (95% CI 0.3-1.2, P=0.001 Bonferroni correction). (Figure 36)

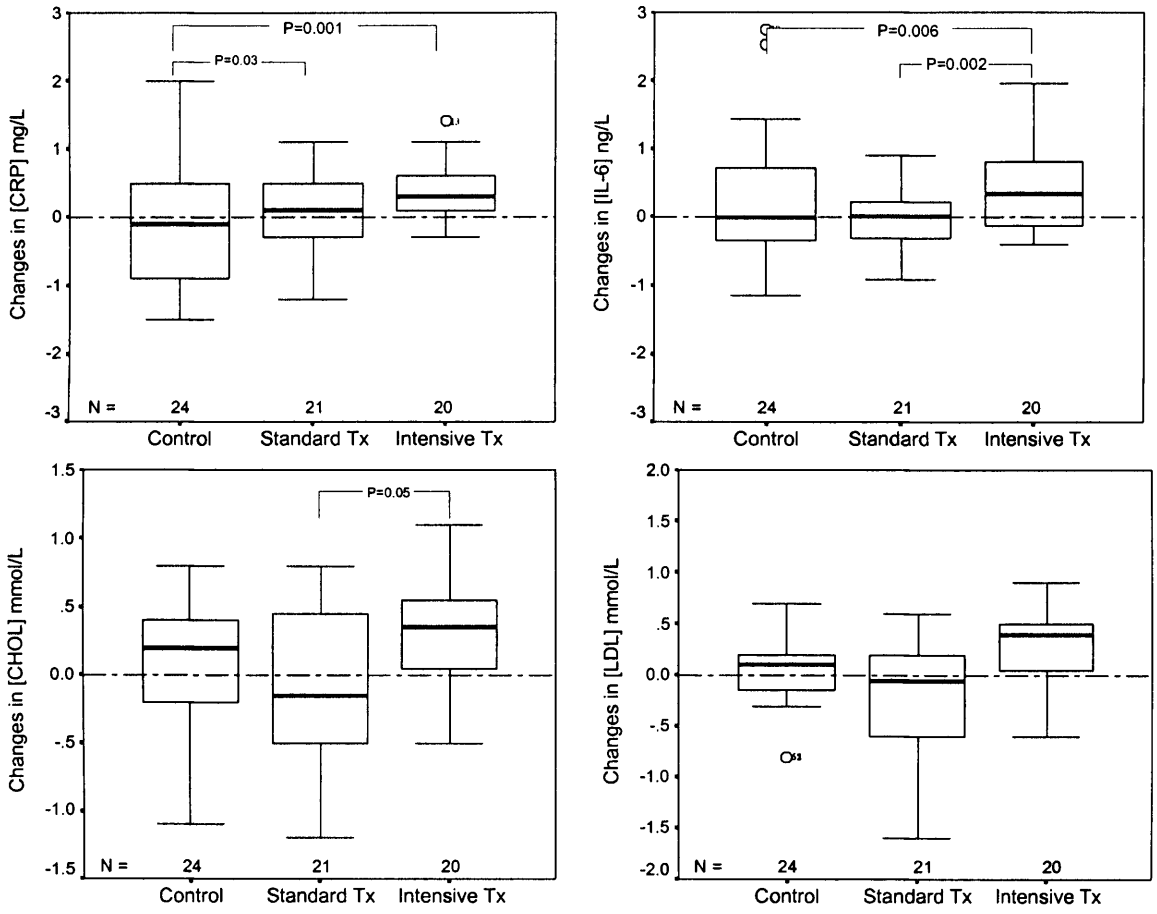


**Figure 36** Box and whiskers plots showing differences in CRP, IL-6, total cholesterol and LDL-cholesterol between baseline and 2 months post-treatment in the 3 experimental groups.
Boxes refer to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations with the subject number. Positive differences indicate a decrease in concentrations at 2 months compared to the pre-treatment baseline. The horizontal dashed line indicates the line of no difference between baseline and 2 months.

The analysis was repeated including as covariates in the model age, gender, body mass index, ethnicity and cigarette smoking. The results remained unchanged with SPT

(P=0.048) and IPT (P=0.002) groups clearly showing a significantly reduced CRP concentration compared to the untreated control at 2 months (Model $R^2$= 0.89). Smoking however approached a significant univariate effect (P=0.057) and therefore we examined whether there was an interaction between treatment category and smoking status. The analysis indicated a significant interaction between the two variables (P=0.019) whereby by post-hoc tests, CRP reductions were statistically significant for both treatment (SPT and IPT) only in the non smoker group (P=0.048 SPT, P=0.011 IPT respectively) (Figure 37) compared to the untreated control, whereas in the smoker subgroup only IPT patients showed a further decrease (P=0.030) compared to the control.

When we examined the differences in IL-6 serum concentrations we found that only the IPT group showed a significant decrease in this marker when compared to the untreated controls (mean decrease 0.5±0.2 ng/L, 95% CI 0.2-0.9, P=0.006) and to the SPT group (mean decrease 0.6±0.2 ng/L, 95% CI 0.2-1.0, P=0.002). These finding were confirmed after inclusion in the analysis of age, gender, ethnicity, body mass index and cigarette smoking which also had a positive interaction with the treatment (P=0.021). Non smokers individuals of the IPT group were those showing significant differences in IL-6 concentrations when compared to the untreated control (P=0.005) and to the SPT group (P=0.002). Box and whiskers plots of the differences in CRP, IL-6, total and LDL-cholesterol between baseline and 2 months for each treatment group are displayed in Figure 36.

Lipid levels did not change significantly among different groups except for total cholesterol. We observed a 0.3±0.1 mmol/L (95% CI 0.01 to 0.6, P=0.05) decrease of total cholesterol in the IPT group when compared to the SPT group (Figure 36). A within group descriptive analysis showed a reduction in total cholesterol of 0.3 mmol/L

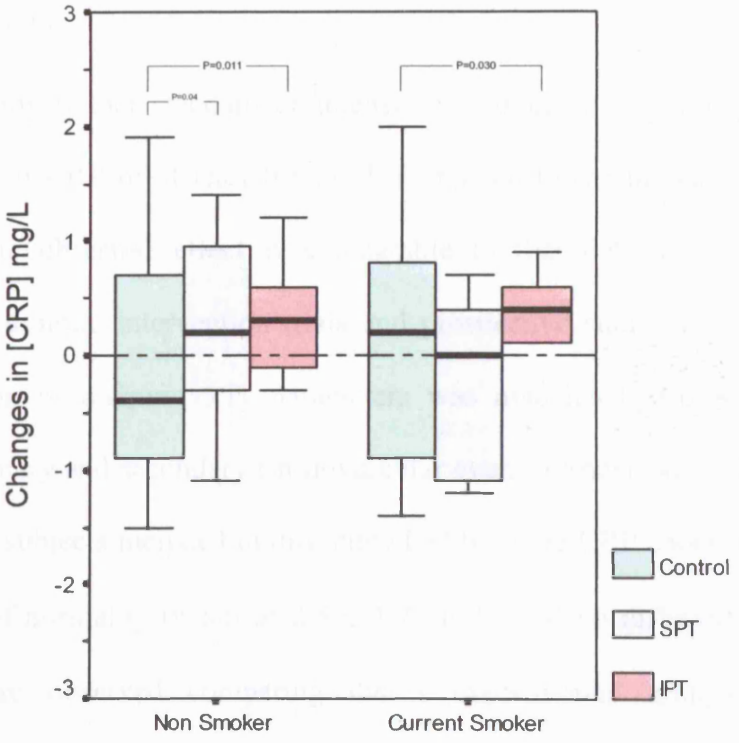


**Figure 37** Box and whiskers plots showing differences in CRP according to smoking status in the 3 experimental groups.
Boxes refer to the 25$^{th}$ (bottom) and 75$^{th}$ (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations with the subject number. Positive differences indicate a decrease in concentrations at 2 months compared to the pre-treatment baseline. The horizontal dashed line indicates the line of no difference between baseline and 2 months.

(95% CI 0.1 to 0.5, P=0.004) in the IPT group only and this was accompanied by a decrease in LDL of the same magnitude (0.3 mmol/L, 95% CI 0.05 to 0.5, P=0.019).

## 6.5 DISCUSSION

Periodontal therapy (either standard or intensive) resulted in a significant additional reduction in serum CRP of at least 0.5 mg/L compared to the untreated control. The magnitude of the observed effect is comparable to that detected in a variety of cardiovascular antibiotic intervention trials and prospective statins trials where drug-induced reduction of inflammatory parameters was associated also with a reduced frequency of primary and secondary cardiovascular events (Anderson et al. 1999; Stone et al. 2002). The subjects included in this study had baseline CRP concentrations in the upper quartiles of normality (mean of 2.5 ± 1.7 mg/L) and no differences in possible confounders were observed comparing the 3 experimental groups (Table 33). Importantly during the study no significant changes in lifestyle, habits, medical health or medications were detected. This indicates that severe, generalized periodontitis in otherwise healthy individuals contributed to the systemic inflammatory burden in this population. Proposed mechanistic explanations include: i) the local, infection-driven production of inflammatory mediators, primarily interleukin-1 and 6, in the diseased periodontium may be dumped into the systemic circulation (Graves 1999; Offenbacher et al. 1981); ii) the ability of periodontal pathogens and/or their toxins to gain access to the systemic circulation and thus induce an inflammatory response (Haraszthy et al. 2000; Herzberg & Weyer 1998); and iii} a combination of the above.

The present results were obtained in a proof of principle randomized controlled clinical trial in a population with advanced, widespread periodontitis representing the more severely affected, but otherwise systemically healthy, periodontal patients. The interest in this group comes from our previous observation (Chapter 4) that these may be the subjects presenting with the most significant periodontitis-associated systemic inflammatory burden. These results, however, do not allow generalization of the results

to periodontal patients suffering from less severe and/or more localized forms of disease. Data will have to be confirmed and expanded in larger trials to better understand what proportion of the 10-15% of subjects suffering from severe periodontitis have increased systemic inflammation as a result of this chronic infection.

Periodontitis is an infection caused by gram negative bacteria that are organized in a biofilm in a sub-gingival location between the diseased root surface of the tooth and the junctional epithelium of the gingiva. As a chronic biofilm-centred infection, it is relatively insensitive to the effect of systemic antibiotics and its treatment requires, in the first instance, the removal of the biofilm on the root surface by mechanical professional instrumentation. Comparing the results of our pilot intervention cohort where we found a dose dependent effect in terms of reduction of systemic inflammation based on the degree of local response of periodontal therapy (Chapter 4), the standard treatment group in this trial showed a better response after two months of therapy. This observation could be explained because in this study we compared CRP changes between the standard and the untreated control group whose subjects showed an increased concentration of CRP after two months. We observed, therefore a significant difference in serum CRP concentrations that would have not been detected within the SPT group if we had not enrolled a control population.

In the IPT group, local application in the periodontal pockets of a controlled release formulation of minocycline was utilized to supplement the mechanical action of scaling and root planing. Such application allowed the achievement and maintenance of high local concentrations of antibiotic at the site of infection (the periodontal pockets) without reaching detectable levels of minocycline in the serum (Paquette & Santucci 2000). Previous investigations have indicated that the adjunctive use of controlled delivery antibiotic formulations in the periodontal pockets lead to better control of periodontal infections and reduction in gingival inflammation (Pavia et al. 2003).

Interestingly, the adjunctive periodontal effect of the application of minocycline microspheres was particularly evident in smokers (Paquette et al. 2004; Paquette et al. 2003), an observation that agrees well with the current one that IPT but not SPT was effective in decreasing systemic inflammation in smokers. Although a possible systemic effect of the antibiotic cannot be completely ruled out, the facts that i) the total applied dose was 80±25 mg per patient; ii) the antibiotic was delivered from the controlled delivery platform over a 21-day period; and iii) minocycline concentrations in serum have been shown to remain below detection level (Paquette et al. 2004) after standard periodontal treatment, make it unlikely that the observed effect may have been due to a systemic effect of minocycline. Within the limitation of this analysis it appears that the intensive treatment approach enabled the host to re-establish a better homeostatic status compared to the standard therapy. A significant reduction in systemic IL-6 concentration, which is known to be produced locally in the diseased periodontium and is the main inducer of the acute phase response (See Section 1.1.3), was detected only in the IPT group.

As indicated, this study was designed to detect changes in CRP concentrations only. A serious limitation of theses preliminary results lies in the small number of subjects included.

The baseline CRP and IL-6 concentrations although not statistically significantly different appeared somehow unbalanced between the three study groups. We cannot exclude that such imbalance, other confounding factors and the limited number of individuals affected the final outcome of the trial itself. We are confident however of the validity of our results since the outcome of a multivariate analysis including the main determinants of CRP serum concentrations (age, gender, body mass index) did not undermine our conclusions. Further strengths come from the observation that cigarette smoking appeared to be the only negative influential factor. There has been a lot of

debate on the role of smoking as the major confounder in the association between periodontitis and systemic health. Our data clearly indicate that smoking affects individual responses to periodontal therapy not only resulting in a less good clinical outcome as several reports have shown (Tonetti 1998), but also producing a less good systemic host response (Fredriksson et al. 2002; Hyman et al. 2002). The fact that smokers' CRP concentrations responded to IPT and not SPT treatment supports the notion that periodontitis contributed to the increased inflammatory burden of the smokers as well. Further research in this matter is however needed.

Furthermore the exploratory analyses of the effects of periodontal therapy on cholesterol levels deserve some discussion. No changes were observed comparing baseline and 2 months levels of these parameters in the control and the standard treatment groups. The intensive periodontal therapy group, on the other hand, showed a significant reduction in total cholesterol compared to the standard therapy group but not the untreated control. Descriptive analysis showed that some reductions of total and LDL cholesterol were present within the IPT group.(Figure 36).

We cannot exclude that pure chance might stand behind these findings even though a biological plausible effect of periodontal infections on the metabolic state of an individual has been validated through a series of investigations. A significant association between periodontitis and cholesterol has been already reported (Katz et al. 2002; Wu et al. 2000b). Other chronic infections (*C. pneumoniae, H. piilory*) have been associated with increased concentrations of plasma cholesterol and triglycerides (Laurila et al. 1997). Several reports have also indicated that the acute phase response induces lipaemia (Khovidhunkit et al. 2000). LDL and total cholesterol are thought to be increased in chronic inflammatory conditions due to the recognized scavenging effect of lipoproteins on bacterial components (primarily endotoxin) (Harris & Kasravi 2003). These data support the hypothesis that patients suffering from severe

periodontitis, subsequent to recurrent phenomena of endotoxaemia and/or bacteraemia, experience a similar metabolic change (lipemia). The inflammatory local production of cytokines (IL-1,TNF-α) and its effect on other systemic mediators (IL-6) might induce alterations of lipid metabolism such as increased LDL and triglycerides due to increased hepatic lipogenesis, lipolysis from adipose tissue or reduced blood clearance. (Iacopino & Cutler 2000). Bacterial toxins (LPS) can also induce changes in cholesterol concentrations (reduced HDL and increased LDL) or target glucose metabolism and produce a state of insulin resistance (See section 1.2.1 and 1.2.4). A low-grade state of systemic inflammation can also affect directly insulin production and sensitivity. Recent experimental evidence support this hypothesis indicating that periodontal infection might cause hyper-triglyceridaemia (Uchiumi et al. 2004) and changes in HDL similar to that of an acute phase response (Pussinen et al. 2004). Further investigations are needed to better explore the relationship between periodontitis, periodontal therapy and lipid metabolism.

## CONCLUSIONS

- Chronic severe periodontitis causes a moderate systemic inflammatory state.
- Standard periodontal therapy contain the systemic host response over a two months period whereas an intensive therapy approach, including the use of a locally delivered antibiotic, produces a significant reduction in inflammatory markers compared to an untreated control.
- Age, obesity and smoking act as confounder in the association between periodontal infection and systemic response.
- Intensive periodontal therapy seems to influence lipid profile of an otherwise healthy individual.
- This trial reported short term changes in inflammatory markers. Extension of the observation period is necessary to assess the persistence of the positive treatment effect.

# CHAPTER 7.

# 6 MONTHS EFFECTS OF DIFFERENT PERIODONTAL THERAPY REGIMENS ON SYSTEMIC INFLAMMATORY MARKERS

## A RANDOMISED CONTROLLED CLINICAL TRIAL

## 7.1 SUMMARY

Infectious diseases such as periodontitis might be linked with increased risk of systemic diseases. We investigated whether different periodontal treatment regimens influenced overtime inflammatory and lipid markers. Patients with severe chronic generalized periodontitis were enrolled into a pilot intervention 6 months trial. They were evaluated at baseline, one, two and six months after being randomized to a standard or intensive (including local delivery of an antimicrobial) course of periodontal treatment. Individual who received the intensive regimen showed significant reductions in inflammatory markers at one ($p=0.0406$) and two ($p=0.0060$) months together with an improved metabolic state (2-6 months reduction in lipid markers $p=0.0320$ and $p=0.0432$ respectively). Our findings indicate that periodontitis causes an increased systemic inflammatory burden and an intensive therapy regimen is more effective in re-establishing a more favourable systemic response.

## 7.2 INTRODUCTION

Periodontal infections are commonly thought to have limited influence on general health and wellbeing. In the beginning of last century however physicians hypothesized a possible pathological effect of distant tissue infections ("foci") on the onset and progression of serious systemic diseases (rheumatism, eye problems) (O'Reilly & Claffey 2000). An increasing number of new experimental investigations suggests that periodontal infections may have systemic implications in otherwise healthy individuals (see section 1.3). A plausible biological association between periodontal infections and a series of systemic illnesses (pre-eclampsia, coronary atherosclerotic events, cerebrovascular ischemia, respiratory infections and metabolic syndrome) has been proposed (section 1.3). The chronic infectious-inflammatory burden of periodontitis may represent for the individual a possible threat on other systemic inflammatory diseases (e.g. metabolic syndrome and atherosclerosis). The issue however remains poorly investigated. Oral infections as triggers of a state of chronic systemic inflammation represent one mechanistic explanation. However, it is likely that other important factors (smoking, gender, body mass index, age, socio-economic status) act as confounders on this association. Our previous intervention studies (Chapter 4 and 6) resulted in a positive association between periodontal infections and systemic markers of low-grade inflammation. Standard periodontal therapy showed a positive effect in removing such inflammatory insult only after six months of therapy in a cohort of 94 individuals (Chapter 4). When we evaluated the short term effects of different periodontal therapy regimens comparing the results with an untreated control, data showed that an intensive periodontal therapy regimen was in the short term more effective in controlling the systemic inflammatory burden compared to the standard approach. A secondary analysis of these data indicated a possible effect of the intensive

therapy on other systemic markers (cholesterol). In this investigation we therefore intended to further explore the association between severe periodontal infections and systemic health by performing a pilot intervention trial comparing the standard and intensive periodontal therapy approach with a longer follow-up (6 months).

## 7.3 METHODS

### 7.3.1 STUDY DESIGN

40 out of 47 consecutive, eligible otherwise healthy individuals, free from any medical treatment, referred to the EDI and in need of treatment for severe generalized periodontitis gave written informed consent to participate in this investigation. Possible candidates for the investigation were selected and enrolled into the trial according to the inclusion and exclusion criteria described in section 3.1.2 with the additional exclusion criteria for individuals with allergy to tetracyclines. The study protocol had been reviewed and approved by the UCL Hospitals ethics committee.

It was a randomized, single blind, controlled clinical trial of 6 months duration comparing the effects of a standard periodontal treatment regimen (SPT=mechanical removal of subgingival plaque and calculus deposits) with an intensive one (IPT= consisting of SPT and adjunctive local periodontal delivery of minocycline encapsulated in resorbable microspheres – Arestin, Orapharma, PA, USA) (Figure 38). Primary outcome variables were the differences in changes in serum inflammatory and lipid markers between SPT and IPT patients at 1, 2 and 6 months. We randomized subjects using a permuted block approach, stratifying by smoking status. Allocation was concealed to the therapist until completion of the common part of therapy for both the test and control groups with opaque envelopes. All clinical and laboratory measurements were performed in a blind fashion.

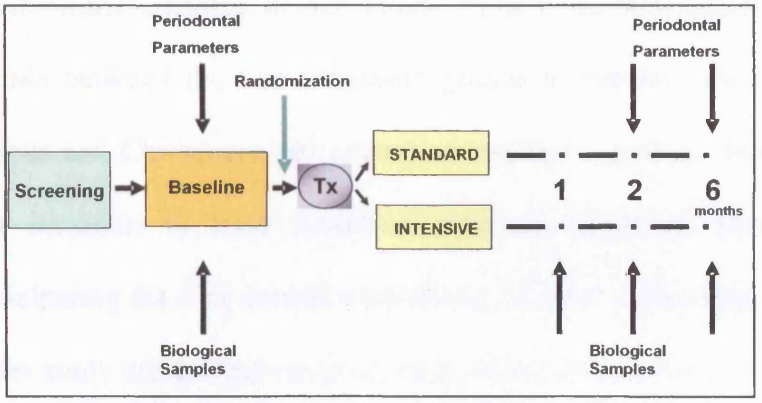


**Figure 38** Study Experimental Design

### 7.3.2 BIOCHEMICAL MARKERS

We collected serial blood samples (see section 3.4 for detailed procedure) before and 1, 2 and 6 months after completion of periodontal therapy and processed them in a blind fashion for CRP (Immunoturbidimetric assay, Cobas Integra, Roche AG Diagnostics, Mannheim, Germany, detection limit 0·25 mg/L, inter- and intra-assay coefficient of variation, CV, of 4% and 5%) and IL-6 serum concentrations (ELISA Quantikine HS, R&D System, Minneapolis, detection limit 0·04 ng/L and inter- intra- assay CV less than 5%). WBC and lipid markers, total (TC) and HDL (HDL-C) cholesterol were also quantified using standard clinical chemistry procedures on an automated analyzer.

### 7.3.3 STATISTICAL ANALYSIS

Data are reported as geometric mean± standard deviation and 95%CI intervals unless otherwise specified. Differences between the two treatment groups at baseline were assessed by t-test for continuous and Chi-square test among categorical variables. We chose a multiple end-point intention to treat statistical approach (O'Brien non parametric rank-sum test) anticipating the non normal distribution of most of the data. This choice was based upon the study design that assessed multiple parameters for each group of factors, e.g. leukocytes, CRP and IL-6 for systemic inflammation. With such design, outcomes are examined within the same patient and therefore are correlated. This global statistical approach uses the within-patient correlation among different outcomes in computing the estimate of the treatment effect and the test statistic (Tilley et al. 1999; Tilley et al. 1996).

In the past multiple outcomes have often been assessed by $T^2$ Hotelling test which evidenced differences in any direction between treatment groups with respect to multiple outcomes (Pocock et al. 1987). Its non specific nature however does not allow discriminating between a favourable or an unfavourable effect of the treatments compared. Bonferroni multiple comparison is probably the most widely used correction

for multiple comparisons (O'Brien & Fleming 1979). O'Brien did report that such analytical approach for multiple endpoints would produce statistical significance just if only one of the endpoints had a significant P statistic ($P<0.01$) but in the end the treatment effect would not be considered significant. Similarly this approach would not result in a significant effect if all analysed endpoints improved equally but no single outcome was highly significant, we would then speculate that the treatment was not effective although each endpoint was positively affected by the treatment. Using a global statistical approach there is no need for investigators to indicate primary or secondary outcomes and it perhaps may represent a research strategy for summarizing and interpreting multiple relevant endpoints in intervention trials. Indeed some antibiotic interventional trials have successfully adopted such a strategy (Anderson et al. 1999; Stone et al. 2002; Wiesli et al. 2002) .

In particular we investigated the treatment effect across a global score for inflammatory and lipid outcomes (CRP, IL-6, WBC, T-C and HDL-C), an inflammatory score (CRP, IL-6, WBC) and a lipid score (T-C and HDL-C). P values less than 0·05 were deemed significant. We also conducted a secondary analysis (Mann-Whitney test) to understand the relative contribution of each individual variable to the global scores (SAS, version 8.2, Chicago, USA, statistical software package).

## 7.4 RESULTS

The baseline characteristics of the experimental population are reported in Table 36. At baseline, we did not observe significant differences in any characteristics of the subjects by treatment group. All subjects had severe generalized periodontitis as indicated by the presence of an average of 79±26 periodontal pockets and 67±14% of sites with bleeding on probing. Peridontal therapy produced significant improvements in both groups (Table 37). No statistically significant differences were found between the two treatment regimens.

**Table 36** Baseline Patient Characteristics.

| VARIABLE | OVERALL(N=40) MEAN±SD (95% CI) | DIFFERENCE BETWEEN TEST AND CONTROL GROUPS P-VALUE † |
|---|---|---|
| Age, years | 48±7 (46·0-50·6) | 0·8281 |
| Gender, Males (%) | 22(55) | 0·7512* |
| Ethnicity, Caucasians (%) | 30(75) | 0·1981* |
| BMI, $Kg/m^2$ | 26±4 (24·4-26·8) | 0·9675 |
| Systolic BP, mm Hg | 134±15 (129·1-138·8) | 0·4329 |
| Diastolic BP, mm Hg | 86±10 (82·9-89·4) | 0·8533 |
| Never Smokers (%)<br>Current & former (%) | 30(25)<br>10(75) | 1·0000* |
| Family history of CVD, (%) | 26(65) | 0·3202* |
| IL-6, ng/L‡ | 1·4±0·9 (1·4-1·9) | 0·2333 |
| CRP, mg/L‡ | 2·0±1·8 (1·9-3·0) | 0·2074 |
| WBC, $10^9$‡ | 6·9±1·9 (6·6-7·8) | 0·2065 |
| Cholesterol, mmol/L‡ | 5·4±0·7 (5·2-5·7) | 0·7252 |
| HDL-C, mmol/L‡ | 1·3±0·4 (1·3-1·5) | 0·6108 |

† Independent t-test (or Chi-Square Test for dichotomous-variables *) comparing SPT(standard periodontal treatment) with IPT(intensive periodontal treatment) subjects ‡ Geometric mean values are reported

**Table 37** Clinical periodontal parameters before and after treatment.

| VARIABLE= MEAN±SD, | BASELINE | | 2 MONTHS | | 6 MONTHS | |
|---|---|---|---|---|---|---|
| | SPT | IPT | SPT | IPT | SPT | IPT |
| PPD | 4,3±0,8 | 4,5±0,8 | 2,9±0,3 | 3,0±0,3 | 3,0±0,4 | 2,9±0,3 |
| REC | 1,0±1,1 | 1,0±0,9 | 1,6±1,2 | 1,8±1,1 | 1,4±1,2 | 1,7±1,2 |
| CAL | 5,4±1,5 | 5,5±1,5 | 4,5±1,3 | 4,8±1,3 | 4,5±1,3 | 4,5±1,4 |
| FMPS | 59,5±20,5 | 52,2±23,2 | 13,9±14,8 | 15,7±13,8 | 15,5±11,7 | 14,5±10,7 |
| FMBS | 68,4±15,9 | 66,4±13,0 | 25,1±8,9 | 19,9±7,9 | 31,7±11,5 | 25,5±11,3 |
| NPPK | 79±29 | 80±25 | 19±12 | 20±11 | 22±12 | 16±9 |

When we examined the global scores, combining inflammation and lipid results, the intensive treatment regimen produced a significantly better effect on systemic parameters than the standard treatment at each time point examined (one, two and six months after therapy) (Figure 39).

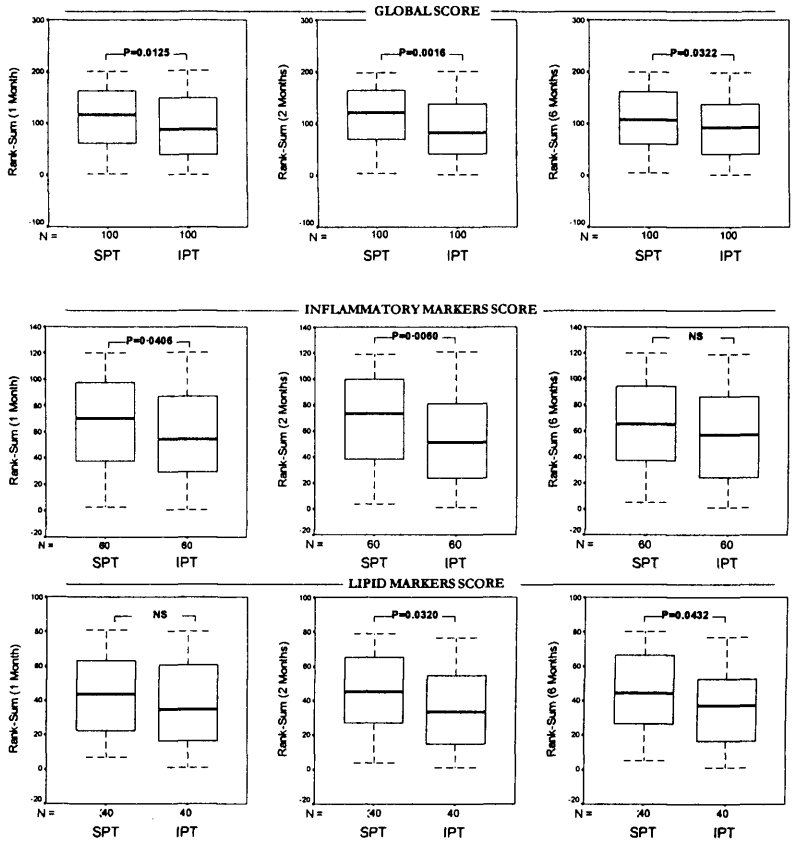


**Figure 39** Rank-sum Score Differences (O'Brien non-parametric test) for global, inflammatory and lipid markers by treatment groups.

We found that patients in the intensive group had significant changes in inflammatory markers at one (weak) and two (strong) months (inflammatory score). The same group also showed a significant change in lipid markers sustained from two to six months after treatment (lipid marker score). Subjects did not show significant changes in body mass index over time (0·13±0·74 difference at two and six months, p=ns) nor reported any change in diet, habits or medical treatments. The IPT regimen resulted in significantly better changes in IL-6 concentrations than SPT at one and two months (0·43±0·23 ng/L difference Baseline-1 Month, 95%CI 0·03-0·88 p=0·0284, 0·34±0·20 ng/L Baseline-2 Months, 95%CI 0·07-0·76 p=0·0284 respectively)(Figure 40-A). Similar results were observed for CRP (0·41±0·20 mg/L difference Baseline-2 Months, 95%CI 0·01-0·82 p=0·0438)(Figure 40-B) and total cholesterol (0·32±0·14 mmol/L difference Baseline-2 Months, 95%CI 0·04-0·60 p=0·0254) at two months only (Figure 40-C).

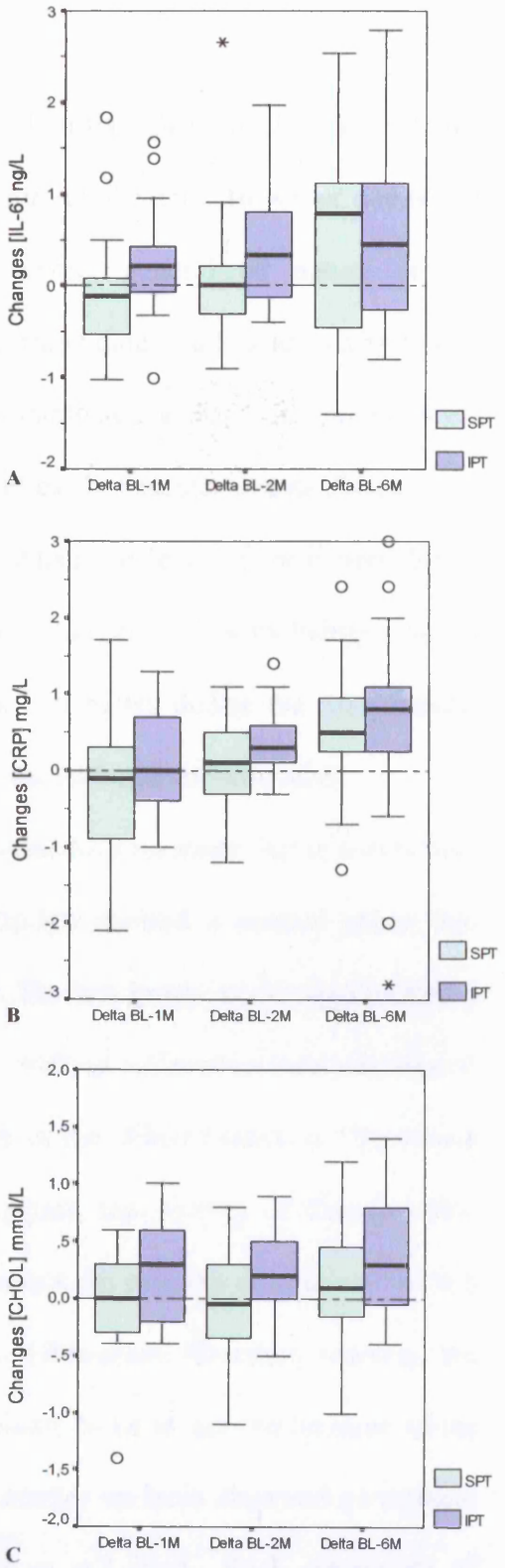


**Figure 40** Box and whiskers plots showing changes in IL-6 (A), CRP (B) and Cholesterol (C) between standard (SPT) and intensive (IPT) periodontal therapy
The box refers to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers whereas asterisks stand for extreme observations. The horizontal dashed line indicates the line of no difference.

## 7.5 DISCUSSION

Our results indicate that severe generalized periodontitis causes a chronic systemic inflammatory response and possibly changes in serum cholesterol. However due to the small number of patients examined and the severe, generalized nature of the periodontitis we would urge caution in generalizing these data to all patients affected by periodontitis. Previous association studies have established a dose dependent effect between the extent and severity of periodontitis and cardiovascular events (Slade et al. 2000; Slade et al. 2003). This study does not establish the level of local periodontal infection that is capable of having a systemic effect. Larger studies including a wider spectrum of disease presentation will be necessary to better define the periodontitis subjects in whom the local infection causes significant systemic inflammation.

With regards to the magnitude of the systemic inflammatory response that is attributable to periodontitis in this population, the present design utilized a control group that represents the current standard periodontal therapy. The test group received an intensive periodontal therapy regimen that consisted of SPT with an adjunctive local delivery of minocycline microspheres in all periodontal pockets of the affected subjects. The choice of this design was largely due to ethical considerations: the severity of disease in the population required delivery of standard care to assess the primary outcomes over a 6 month period. Differences between the two groups in this study, therefore, represent the added benefit of the adjunctive antibiotic and are likely to be an underestimation of the probable effect of periodontal therapy. In previous studies we have observed a treatment associated decrease in CRP ranging between 0.4 to 0.7 mg/L. Such magnitude of change was essentially identical in a cohort design comparing 6 month follow up with baseline values (chapter 4) and in the short term randomised controlled clinical trial described in chapter 6 comparing changes observed in the untreated control and IPT.

In the context of the two previous intervention studies by our group, these data also provide considerable insight into the kinetics of change observed following different regimens of periodontal therapy. Periodontal therapy successfully reduces the individual systemic inflammatory burden already after 2 months from therapy. The use of locally delivered antimicrobials may accelerate the resolution of the infectious and inflammatory burden in the diseased periodontium (see the presence of significant differences already at 1 and 2 months with IPT compared to the improvements in SPT observed by 6 months). At 6 months, however, even a standard treatment approach would significantly re-establish a systemic homeostatic state.

In this investigation we aimed at exploring the effect of periodontitis on a cluster of systemic inflammatory markers including WBC and lipid levels. We chose a global statistical approach to ascertain whether the treatment used had any impact on these markers. CRP and IL-6 serum concentrations are very sensitive and reliable markers used to assess the individual systemic inflammatory burden (See section 1.1). Both markers have also been independently defined as good predictors of future developments of serious systemic condition (coronary artery events, insulin resistance and type-2 diabetes, hypertension). WBC is known as a crude marker of systemic inflammation and it correlates well with the host response to a variety of stimuli (Da Silva et al. 1995) . This marker has also been associated with a significant prediction of future cardiovascular events and glucose intolerance in different populations (Brown et al. 2004; Haim et al. 2004; Ohshita et al. 2004). Independent of which therapy was performed, individuals in our study showed a significant reduction of WBC already after 1 month and such effect remained constant up to six months after therapy. No differences however were noted between treatment regimens.

Lipid markers such as total cholesterol, HDL have assumed crucial importance when it comes to predict individual future risk for cardiovascular events (Stamler et al. 1986).

Changes in concentrations of these markers however have also been associated with acute and chronic infections (Leinonen & Saikku 2002). Our data suggest that severe periodontitis may represent one triggering factor of lipid levels alterations. Whether this finding is true or confounded by other important factors (diet, smoking, age) can only be ascertained by re-addressing the question in further clinical intervention trials.

The observation that circulating levels of inflammatory markers (CRP, IL-6, WBC) as well as cholesterol might be influenced by a chronic oral infection may have important clinical consequences. First, since inflammation plays an important role in the pathophysiology of various conditions (metabolic syndrome, blood pressure, vascular health) the association of mild chronic inflammation with future serious events (cardiovascular, ischemic) in observational studies could be influenced by an underlying severe periodontal infection.

Second, if the inflammatory processes associated with these serious events are caused by yet unknown noxious stimuli, severe periodontal infections might contribute to exacerbate the inflammatory burden of the affected individual and thus participate in the pathophysiology of chronic inflammatory disease such as atherosclerosis or metabolic syndrome.

Third, if periodontitis were the major inflammatory stimulus in at least some periodontitis patients, severe periodontal infections may represent a major etiologic factor for atherosclerosis, metabolic syndrome and their sequelae.

The significance of periodontitis as a cause of systemic inflammation and potentially disease has to be discussed in the context of the high prevalence of chronic periodontitis which affects in mild forms up to 40% and in more severe forms a good 10% of the adult population (Albandar & Rams 2002; Papapanou 1996; Papapanou 1999). The significance of recognizing this source of chronic inflammation comes also from the nature of the infection: periodontitis is unique since it is a biofilm centred infection that,

as such, is not responsive to systemic antibiotics treatment in the absence of specific local measures to disperse the biofilm. In this respect the presence of periodontitis can also represent a confounder for the assessment of the health benefits of the ongoing intervention trials based on the administration of systemic antibiotics to eliminate undetected chronic infections.

## CONCLUSIONS

- Intensive periodontal therapy produces a significant effect on a cluster of inflammatory and lipid markers compared to a standard treatment approach.
- A significant anti-inflammatory effect (reduction in IL-6 and CRP) is exerted as early as 1 and 2 months after therapy.
- These improvements in inflammatory parameters are sustained for at least 6 months after periodontal therapy.
- A concomitant reduction in total cholesterol is observed after 2 and six months.
- The results of this trial extend and are consistent with those reported in Chapter 6.

# CHAPTER 8.

# GENERAL DISCUSSION AND CONCLUSIONS

The results of the 3 reported intervention trials demonstrated that periodontitis causes systemic inflammation. Periodontal therapy resulted in significant improvements of the local periodontal condition: decreases in infection with marker periodontal pathogens and in local inflammation were observed.

The novelty and importance of such findings lie in the biologically plausible hypothesis that sustained periodontal infections triggering a systemic low-grade inflammation may produce systemic complications (atherosclerosis, type-2diabetes, pre-eclampsia, PBLW delivery).

APR is a rapid and effective host defensive mechanism mounted to clear a variety of tissues' insults. In most of the cases this defence is successfully accomplished without complications. However in case the insult persists, repetitive stimulation of this host innate response may be harmful to the host. An exhaustive body of literature support the pathogenetic role of chronic inflammation, assessed by small but significant increases of specific markers (CRP, IL-6), on the onset of serious systemic illnesses (hypertension, insulin resistance, type-2 diabetes, metabolic syndrome and atherosclerosis)(See chapter 1 for more details).

As a result of periodontal treatment patients with severe generalized periodontitis displayed a clinically relevant decrease in serum inflammation (and CRP in particular). The magnitude of the observed improvement was in the range of 0.5 mg/L (cohort design) to 0.8 mg/L (intensive periodontal therapy compared to the untreated control). The observed size of the effect and the consistency across the trials add validity to the results. A "real" effect of this scale is consistent with a change in CRP associated cardiovascular risk class, an excellent long term predictor of cardiovascular events and diabetes onset (Ridker et al. 1998; Ridker et al. 1999; Pearson et al. 2003; Yudkin et al. 2004).

Moreover the observed improvements could be maintained for up to 12 months without additional periodontal therapy indicating that, in the presence of supportive periodontal care at 3 month intervals as delivered in standard practice, the benefits could be sustained for a sufficient period to observe potential general health benefits.

Taken together, the data reported in these trials indicate that to achieve a significant serum outcome, a clinical significant response in terms of resolution of the local infectious and inflammatory parameters was necessary. This may explain the controversial results reported in previous intervention trials where the clinical outcomes of periodontal therapy did not achieve the expected improvements.

In our first pilot trial we were able to observe a statistically significant reduction in serum inflammatory markers after standard periodontal therapy only six months after completion of therapy. Significant added benefit in serum response was observed with the adjunctive application of minocycline microspheres. Its adjunctive effect could not be fully accounted for by the differences in clinical periodontal outcomes; this supports the hypothesis that periodontal therapy needs to be optimised to achieve systemic benefits. Our data indicate that an intensive regimen including adjunctive local delivery of minocycline may be more suited than classical mechanical instrumentation alone: bigger decreases at earlier time points were observed following delivery of this form of treatment. This notion is consistent with the recognised adjunctive benefit of local controlled delivery of antibiotics in terms of better suppression of the periodontal infection and inflammation. The data indicating that IPT provided benefits to both smokers and non-smokers, while SPT did not provide significant changes in the smoking population merits attention: IPT has been known to achieve better control of periodontitis in smokers. Further research however is needed in order to explore the potential additional effect of locally delivered or systemic antibacterial and/or anti-inflammatory medications in different populations presenting with a wider spectrum of

severity of periodontal infection. Moreover these data indicated the need of novel intra-oral clinical or biochemical parameters which better define the potential systemic inflammatory burden of periodontal infections.

The changes in cholesterol levels preliminarily detected in the two randomised controlled trials are noteworthy. Even though these trials were not designed to specifically address this question, since we observed an improvement in the lipid markers in both trials we would raise the hypothesis that periodontal infections may lead to complex metabolic changes (metabolic syndrome) possibly through the increased systemic inflammation. This hypothesis is consistent with previous data indicating that chronic infections may lead to changes in metabolism (Fernandez-Real & Ricart 2003). Changes in cholesterol mediated by the inflammatory response may represent an additional mechanism that would explain the observed increase in carotid intima thickness in periodontitis patients as well as the complex interaction between infection, diet and atherogenesis seen in experimental animal models (Jain et al. 2003; Li et al. 2002; Lalla et al. 2003) .

Having established a causal link between periodontitis and systemic inflammation with a possible effect on lipid metabolism, the next logical step would be to perform large intervention trials for primary prevention of inflammatory-driven systemic illnesses. However it would be advisable to first look at the potential beneficial effect of periodontal therapy on other surrogate parameters (carotid intima thickness, endothelium dependent vaso-dilatation, blood pressure, insulin resistance) and eventually on true cardiovascular and metabolic diseases end-points (fatal and non fatal MI, non-haemorrhagic stroke, glucose intolerance, type-2 diabetes).

Data on the differential performance of the tested periodontal treatment regimens are relevant for the design of intervention trials wishing to establish the nature of the association between periodontitis and systemic diseases. Of interest to this discussion

was also our description of the APR associated with the first week following delivery of periodontal therapy: these acute changes require further investigations in particular in the context of utilising these treatment approaches in subjects with significant systemic pathology such as advanced atherosclerosis, metabolic syndrome or history of cardiovascular events.

Another important aspect emerging from our results is the fact that the risk of having systemic inflammatory sequelae does not seem to be equally shared by the population of patients with severe generalized periodontitis. Specific polymorphisms in the IL-1A, TNF-A and IL-6 genes have been associated with higher concentrations of serum inflammatory mediators after correcting for disease severity and known risk factors. Identification of high risk groups for systemic inflammatory sequelae of periodontitis is an important development. If periodontitis associated inflammation will contribute to causality of major chronic inflammatory disease, these findings on the genetic influences may allow better identification of those periodontitis patients requiring more stringent control of the local infection. Interestingly, these gene polymorphisms have been associated with greater severity of periodontal disease and hence could both contribute to more aggressive disease and more significant systemic sequelae. Furthermore the significance of these polymorphisms is not expected to be limited to the systemic inflammatory response of subjects with periodontitis but should be extended to those observed following the other chronic infections that have been implied in chronic inflammatory diseases.

The extent and severity profile of patients recruited in terms of periodontitis was chosen in order to identify a population with high levels of exposure to periodontal infections. The external applicability of our results to the population of subjects affected with periodontitis requires further studies. A dose dependent effect of periodontitis exposure in terms of both cardiovascular events and systemic inflammatory markers has been

reported. The size of the effect is thought to decrease with a decrease in periodontitis extent and severity; available data however do not allow the establishment of critical levels of disease beyond which systemic implications become more significant. Our data on the relevance of cytokine polymorphisms, furthermore, suggest that such cut-off points may indeed be dependent on the genotype and the environmental exposure profile of the subject.

Individuals are continuously challenged by a vast number of micro-organisms present on the external and internal surfaces of their bodies. The ability to mount a prompt inflammatory response to bacterial pathogens represents an advantage for the innate immune system. Genetic factors play an important role in modulating the individual host response and a series of metabolic pathways have changed during evolution in order to maximise this response to a variety of insults (Fernandez-Real & Ricart 2003). The association between acute phase reactants, insulin resistance and atherosclerotic heart diseases could be considered as the response of the organism to a chronic infectious disease such as periodontitis. Continuous bacterial challenges in addition to a systemic low-grade inflammatory response produce fertile ground for endothelial damage/dysfunction and insulin resistance. Further properly sized and designed clinical trials assessing the relationship between periodontal, cardiovascular and metabolic diseases will answer many of the questions that our results have raised in these areas.

Lastly it seems important to observe that periodontitis is only one of the possible low grade chronic infections that may contribute to this process. The concept of total bacterial and inflammatory burden seems critical to put the role of periodontitis in perspective. After completion of our studies it is possible to state that periodontitis is a cause of systemic inflammation in the studied subjects. At the population level, however, it is perhaps more appropriate to hypothesise that periodontitis contributes to the overall inflammatory burden of the individual. As such, given its high prevalence

and lack of response to systemic administration of antimicrobials, it merits further attention as a possible cause of chronic inflammatory diseases.

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# APPENDIX 1.

University College London Hospitals NHS

NHS Trust

Version 1.2
10-January -03

**Subject Information Sheet**

**A pilot study on the effects of non-surgical periodontal therapy on acute phase serum markers**

**Please read this sheet carefully. Please ask if you do not understand or would like more information**

You are being asked to participate in a research project being conducted with the approval of the Eastman Dental Institute and Hospital Joint Research and Ethics Committee. You have been selected as a potential subject because you have the appropriate oral condition that we are studying. The following information is provided so that you can make an informed decision regarding your willingness to participate.

1 **Background and purpose**

This 1-month clinical study aims to understand better the impact if any, of periodontal disease (gum disease) on general health. In the last ten years considerable evidence has associated chronic, low-grade infections with an increased risk of heart disease. Severe periodontitis is one such infection that has been associated with the risk of heart disease. The nature of this association, however, has not yet been established. Specific protein substances (acute phase proteins) in the bloodstream are known to be risk indicators of heart disease. Infections like periodontal disease may also cause an increase in the blood levels of these proteins. By treating gum disease and maintaining oral health it may be possible to decrease the level of these proteins and thus reduce the risk of heart disease. In this study we wish to monitor changes in these proteins, resulting from initial periodontal therapy, comprised of instruction in oral hygiene techniques followed by thorough scaling and polishing of your teeth in two consecutive appointments.

2. **Procedures**

Approximately 10 patients with periodontitis will participate in this study. You will be asked to make a total of 8 attendances at the Eastman Dental Institute over a period of 2 months.

At the first study visit, a full medical and dental history will be taken. You will be asked to fill in a short questionnaire. Medical signs (weight, height, blood pressure) will be recorded and a periodontal evaluation will be performed. A plaque sample will be collected and a blood sample will also be taken from your arm.

Subsequently, your gum disease will be treated by removal of the tartar, plaque, and bacteria responsible for the disease in two consecutive appointments of about 2 hours each (study visit 2 and 3). At the fourth, fifth, sixth and seventh visit (one, three, five and seven days respectively after the completion of treatment), your oral hygiene will be controlled, you will receive a professional tooth cleaning and a blood sample will be collected and any side effects you may have experienced since last visit will be noted.

At 1 month (eighth visit) after the treatment a periodontal evaluation will be performed and a blood sample taken. Any changes in medications or any side effects you may have experienced since last visit will be noted. This will be carried so we can measure the changes in your periodontal health and in the blood levels of the proteins mentioned above.

3. **Risks and discomforts**

Periodontal probing, scaling and root planing and dental anesthesia injections may involve some discomfort. Blood collection will also cause you some discomfort.

**4. Alternatives**
You do not have to be in this study to be treated for periodontitis (gum disease).

**5. Exclusions**
A history of certain diseases and conditions, or use of certain medications are not allowed in this study. Your study doctor will discuss with you what may exclude you from participating in this study.

**6. Possible Benefits to Participants**
As a result of your improved oral hygiene and the periodontal treatment you will receive during the study, you will have an improvement in your periodontal health. We will compare the improvement of your periodontal condition with the level of the blood proteins analysed, in order to evaluate if reducing the extent and severity of periodontal disease also results in reduction of the blood level of proteins which act as indicators for heart disease.

**7. Research Related Injury**
In the event of personal injury resulting directly from the research procedures, financial compensation cannot be provided. However, every effort will be made to make available to you the facilities and professional skills at the Eastman Dental Institute and Hospital. If complications related to study participation arise, the researchers will assist you in obtaining appropriate medical treatment.

**8. Confidentiality**
The Investigator (study doctor) will make every possible effort to keep your personal information confidential. The results of this research project may be presented at meetings or in publications; however, any research data released or published will not identify volunteers by name.

**9. Whom to Ask Questions Regarding this Study**
You have the right to ask questions concerning this study at any time, and you are urged to do so. You will be informed of any significant new information pertaining to your safety. If you have any questions concerning this study, please contact **Jeanie Suvan** at 020 7915 2335. If you have questions about or would like to report any research related injuries, please contact **Prof. Tonetti** at +44 (0)20 7915 1075.

**10. Voluntary Participation and Right to Refuse or Withdraw**
Your participation in this study is voluntary. You may refuse to participate or may discontinue participation at any time during the study without penalty or loss of benefits to which you are otherwise entitled.

**11. Termination of Your Participation**
Your participation may be ended by the study doctor, without regard to your consent, if you become ineligible to continue in the study, if you fail to follow the study instructions given to you, if you experience a study-related injury, or for any other reason.

# APPENDIX 2.

University College London Hospitals NHS
NHS Trust

Version 1.1 10-January-2003
Centre Number:
Study Number:
Patient Identification Number for this trial:

**CONSENT FORM**

Title of project:

**A pilot study on the effects of non-surgical periodontal therapy on acute phase serum markers**

Name of Principal Investigator: Professor Maurizio Tonetti, DMD, PhD, MMSc, FDS RCPS

**Please initial box**

1. I confirm that I have read and understood the information sheet dated 10.01.2003 (version 1.2) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. I understand that the samples taken from me may be stored and used for the purpose of research. I understand that these results will also remain anonymous. ☐
4. I agree to take part in the above study ☐

| Name of patient | Date | Signature |
|---|---|---|
| Name of Person taking consent (if different from researcher) | Date | Signature |
| Researcher | Date | Signature |

UCL HOSPITALS

UCL Hospitals is an NHS Trust incorporating the Eastman Dental Hospital, Elizabeth Garrett Anderson and Obstetric Hospital, Hospital for Tropical Diseases, The Middlesex Hospital, National Hospital for Neurology & Neurosurgery and University College Hospital.

# APPENDIX 3.

**Stock1**

| | |
|---|---|
| Dibasic potassium phosphate | 0.6 g |
| Distilled water | 100 ml |

**Magnesium Sulphate Stock Solution**

| | |
|---|---|
| Magnesium sulphate | 2.5 g |
| Distilled water | 100 ml |

**Stock Solution 2**

| | |
|---|---|
| Potassium Chloride | 1.2 g |
| Ammonium Sulphate | 1.2 g |
| Monobasic potassium sulphate | 0.6 g |
| Magnesium sulphate stock | 1 ml |
| Distilled water | 99 ml |

**Sodium Carbonate Solution**

| | |
|---|---|
| Sodium Carbonate | 0.8 g |
| Distilled water | 10 ml |

**100 ml of RTF**

| | |
|---|---|
| Stock 1 | 7.5 ml |
| Stock 2 | 7.5 ml |
| Sodium Carbonate | 0.5 ml |
| Distilled water | 80 ml |

Solutions are autoclaved at 121°C for 15 minutes and allowed to cool. A filter-sterilized solution of dithiothreitol (Sigma, 0,02 g in 5m of distilled water) is added.